CELL CYCLE REGULATORY GENES AS MARKERS OF OUTCOME IN SEROUS EPITHELIAL OVARIAN CANCER

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Declaration

"I hereby declare that this submission is my own work and to the best of my knowledge it contains no materials previously published or written by another person, nor material which to a substantial extent has been accepted for the award of any other degree or diploma at any educational institution, except where due acknowledgement is made in the thesis. Any contribution made to the research by others, with whom I have worked at the Garvan Institute of Medical Research or elsewhere, is explicitly acknowledged in the thesis. I also declare that the intellectual content of this thesis is the product of my own work, except to the extent that assistance from others in the project's design and conception or in style, presentation and linguistic expression is acknowledged."

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Abstract

Ovarian cancer affects 1 in 70 women in developed countries and is the leading cause of death from gynaecological malignancies. Ninety percent of ovarian tumours are of epithelial origin. Serous cystadenocarcinoma is the most prevalent of these tumours, constituting 60% of epithelial ovarian cancers. Although, clinical and pathological features of ovarian tumours are currently used to predict the course of disease, there is a need to identify molecular markers that could reliably predict the clinical outcome of patients. Dysregulation of cell cycle control, in particular G_1 to S phase transition, has been implicated in the pathogenesis of many human cancers. Recent studies of molecular markers and clinical outcome in ovarian cancer, have identified several cell cycle regulatory genes that may be important in disease progression that include p53, p21$^{Waf1/Cip1}$ and p27^{Kip1}, although the results are far from conclusive.

In this study the relationship between the expression of p53, p21$^{Waf1/Cip1}$, p27^{Kip1}, p16^{Ink4a}, cyclins D1 and E, and pRb with disease outcome and other clinicopathological characteristics of serous epithelial ovarian cancer was sought. Molecular markers predictive of reduced disease-specific survival (DSS) in the cohort included overexpression of cyclin D1 (p=0.03) and p53 (p=0.02), reduced expression of p27^{Kip1} (p=0.05) and loss of p21$^{Waf1/Cip1}$ (p=0.02), with the latter three being prognostic for a shorter progression-free interval (PFI). When various combinations of p53 and p21$^{Waf1/Cip1}$ expression were analysed, patients whose tumours displayed overexpression of p53 with concurrent loss of p21$^{Waf1/Cip1}$ had a significantly shorter DSS (p=0.0008) and PFI (0.0001) than patients with other combinations of these two molecules. On multivariate analysis overexpression of cyclin D1 and combined loss of p21$^{Waf1/Cip1}$ in the presence of p53 overexpression were independent predictors of DSS when adjusted for volume of residual disease, presence of ascites and performance status. Similarly, loss of p21$^{Waf1/Cip1}$ in the presence of p53 overexpression was independently predictive of a shorter PFI when considered with residual disease, performance status and ascites. Overexpression of cyclin E and p53, reduced expression of p27^{Kip1}, and loss of p21$^{Waf1/Cip1}$ were associated with increasing tumour grade. Expression of pRb, p16^{Ink4a} and cyclin E were not associated with clinical outcome. A more detailed analysis of p53 staining in this cohort of tumours revealed a bimodal distribution of p53 expression. Survival analysis of subgroups of patients when stratified for p53 expression, demonstrated that p53 expression of >5% and <50% was associated with a significantly reduced 5-year DSS (p<0.0001) and PFI (<0.0001), in contrast to patients with tumours displaying p53 expression of <5% or >50%.

In conclusion, cyclin D1 overexpression was associated with poor clinical outcome. Overexpression of p53 with concurrent loss of p21$^{Waf1/Cip1}$ was a stronger prognostic marker of disease outcome than expression of either molecule alone and may be of clinical utility in treating patients with serous ovarian cancer.

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Abbreviations

95% Cis	95% confidence intervals
BSA	bovine serum albumin
B	brenner
CC	clear cell
CDK	cyclin dependent kinase
CDKI	cyclin dependent kinase inhibitor
DNA	deoxyribonucleic acid
DSS	disease specific survival
E	endometrioid
EOC	epithelial ovarian cancer
FIGO	International federation of gynaecology and obstetrics
G_1	gap 1 phase of the cell cycle
G_2	gap s phase of the cell cycle
GOG	gynaecology oncology group
H_2O_2	hydrogen peroxide
IHC	immunohistochemistry
kDa	kilodaltons
LOH	loss of heterozygosity
LN	lymph node
Misc	miscellaneous
Mix	mixed
M	mucinous
NHS	normal horse serum
NS	not specified
PFI	progression free interval
PLAP	placental alkaline phosphatase
PS	performance status
PBS	phosphate buffered saline
pRb	retinoblastoma protein
$p16^{Ink4a}$	16 kDa cyclin dependent kinase inhibitor
$p21^{Waf1/Cip1}$	21 kDa cyclin dependent kinase inhibitor
$p27^{Kip1}$	27 kDa cyclin dependent kinase inhibitor
p53	53 kDa cyclin dependent kinase inhibitor
RD	residual disease
RNA	ribonucleic acid
Ref	reference
S	serous
S phase	DNA synthesis phase of the cell cycle
TBS	tris buffered saline
U	undifferentiated
WHO	world health organisation
χ	chromosome

Chapter One   Clinical aspects of ovarian cancer

1.1   Introduction

Ovarian cancer affects one in seventy women in developed countries, and is the fifth leading cause of cancer-related mortality amongst women (1). Typically this cancer has an insidious onset and over 70% of women present with advanced disease that has spread beyond the ovary. In this population, 5-year survival is 25% despite optimal surgery and aggressive chemotherapy (2).

Treatment for ovarian cancer was first reported in 1586, and by the late 19th century surgical removal of the diseased organ had become accepted practice (3). In addition to the various modifications of surgical practice, the past 30 years have seen the emergence of chemotherapy and radiotherapy as powerful adjuvant tools in the treatment of cancer *per se*.

With an emphasis on centralisation of cancer services and recruitment of patients on well-constructed clinical trials, significant inroads are being made to improve the overall and palliative outcome of patients with ovarian cancer. In addition, a more comprehensive understanding of cancer aetiology is essential to achieve significant advances in the treatment of this disease, and the past decade has seen a flourish of activity in the fields of cancer genetics and molecular biology.

The primary aim of this thesis is to identify a pattern of gene expression in the development of ovarian cancer, which correlates with an aggressive phenotype, and identify biomarkers of clinical utility and therapeutic importance in ovarian cancer. In order to do this it is necessary to review the genetic factors that may predict cancer development and progression, as well as clinicopathological parameters that influence disease outcome once diagnosed. Many tumour types have been identified to involve the ovary, and this thesis concentrates primarily on serous cystadenocarcinomas.

1.2 Clinical epidemiology and risk factors of ovarian cancer

1.2.1 Epidemiology

Ovarian cancer is the leading cause of death from gynaecological malignancies in the western world (4). In 1997, the Australia Cancer Society reported 1151 new cases of ovarian cancer and 740 deaths. This translates to an annual incidence of 11.0 per 100,000 population, and a mortality rate of 6.8 per 100,000 population (5). In Australia there has been no significant change in the incidence of, and mortality rates from ovarian cancer since 1983 (6), (Figure 1.1). The peak incidence of invasive malignancy is in the sixth decade, with the age-specific incidence rising precipitously from 20 to 80 years and subsequently declining (5). The clinical outcome of patients diagnosed with this disease is poor. In over two-thirds of women, presentation is generally delayed and advanced disease diagnosed due to the absence of early signs and symptoms, and a lack of effective screening tests for diagnosing early disease (7). Consequently, the 5-year survival in this group of patients with advanced disease is 25%. Table 1.1 illustrates the distribution and approximate 5-year survival of women diagnosed with epithelial ovarian cancer in developed countries. Distribution of the disease among patients is based on a standardised staging system introduced by the International Federation of Gynaecology and Obstetrics (8). The staging system is discussed in more detail in section 1.3, but in essence, separates women depending upon the spread of the disease at presentation. Treatment of ovarian cancer relies on radical surgery and aggressive cytotoxic chemotherapy, but despite all efforts, the cancer continues to progress rapidly in 15% of patients, and a further 50% suffer relapse within 2 years of their initial treatment.

Ninety to ninety-five percent of ovarian cancer is sporadic in nature, with the remainder being familial (9). Of those sporadic cases, 90% are defined as Epithelial Ovarian Cancers (EOC), denoting their origin from the surface epithelium of the ovary or from its corresponding invaginations into the ovarian stroma (10).

Figure 1.1   Incidence and mortality rates for ovarian cancer in Australia
(Adapted from Reference 2)
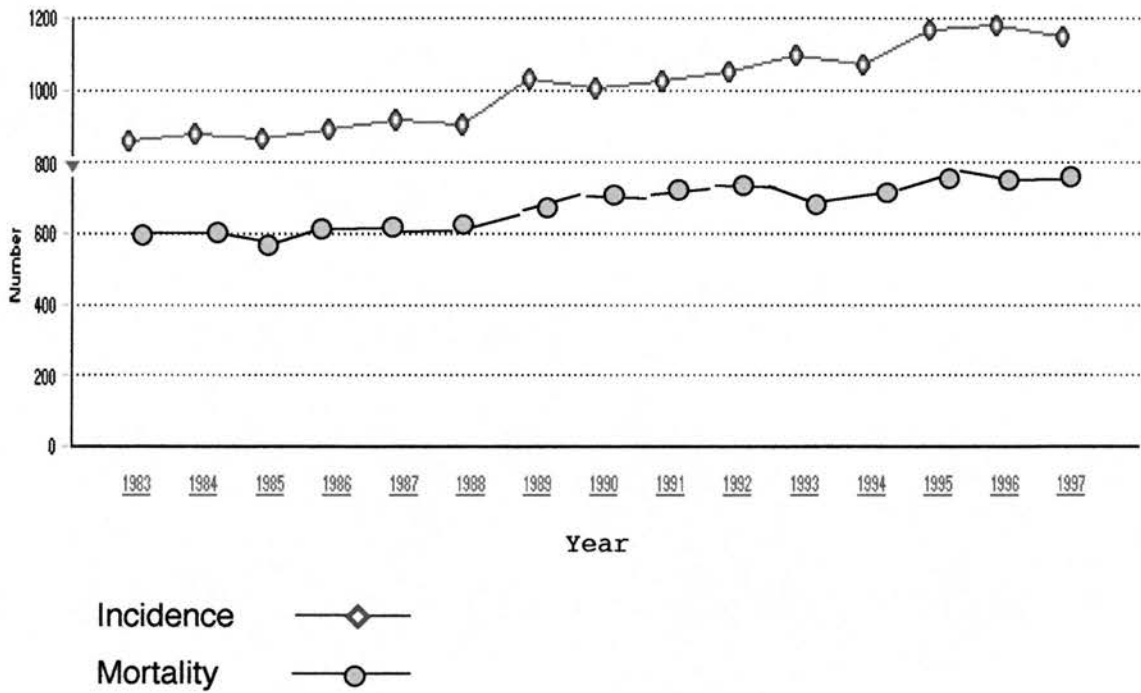
Table 1.1   Five-year survival rates of epithelial ovarian cancer by
FIGO stage (Adapted from reference 7)

Stage	Comment (abbreviated)	Distribution (% population)	5-year Survival (%)
I	Confined to ovary	**24**	**79**
IA		13	84
IB		3	79
IC		8	70
II	Confined to pelvis	**13**	**57**
IIA		3	61
IIB/IIC		10	56
III	Confined to abdomen cavity	**47**	**23**
IIIA			49
IIIB			32
IIIC			19
IV	Metastases elsewhere	**16**	**8**

1.2.2 Risk factors: Sporadic ovarian cancer

The aetiology of sporadic ovarian cancer is unknown. However, epidemiological studies identify numerous factors that may influence the risk of developing ovarian cancer (2, 11-19). The number of ovulatory cycles in a woman's reproductive lifetime is the most significant of these associations, giving rise to the "incessant ovulation" hypothesis (20). Theoretically, the repetitive disruption and repair of the surface epithelium of the ovary during ovulation may lead to spontaneous genetic mutations that in turn may confer an oncogenic phenotype to the epithelial cells. Consequently, reducing the number of ovulatory cycles may result in a decreased risk of developing this disease. Use of the oral contraceptive pill is associated with up to a 70% reduction of developing EOC after 10 years or more of use compared with no-use (11, 12). In addition, multiparity, breast feeding, late menarche and early menopause, that are all associated with reducing the number of ovulations, have been shown to confer a protective effect in developing EOC (13, 15).

Although several other factors have been thought to influence the risk of developing EOC, the findings have generally been inconclusive. The associated risk with the use of perineal talcum powder and EOC has been investigated and remains controversial (21-23). The initial impetus to study this substance was its chemical similarities to asbestos and frequent use of talc around the perineum (24). Asbestos was shown to cause ovarian epithelial hyperplasia in animal experiments, comparable to changes seen in early EOC in patients (24). Heller and colleagues demonstrated retrograde deposition of talc particulates on the ovary through the reproductive tract, although subsequent studies failed to conclusively prove its role as a risk factor for EOC (23, 25, 26). Partly within the same paradigm of retrograde peritoneal exposure to carcinogenic agents, the effect of tubal sterilisation and hysterectomy has been investigated. Green and colleagues reported a significant reduction in the risk of developing EOC by 39% after tubal occlusion and by 36% after hysterectomy in a sample population of Australian women (16). However, surgical interruption of the utero-ovarian circulation results in fewer ovulations, and this may be accountable for the protective effect against EOC (27).

The effects of infertility and use of fertility drugs with in-vitro fertilisation have also been studied (18, 28-31). There is no conclusive evidence that transient use of fertility drugs influence the risk of EOC although, unexplained infertility *per se* may be associated with an increased risk over and beyond that in nulliparous women. The reason for this is unclear. More recently, epidemiological data suggest a 2-fold increase in the risk of developing EOC with prolonged use of oestrogen replacement therapy in the menopause (17, 32). One possible mechanism accounting for this increased risk is the direct effect of oestrogen on epithelial ovarian cells. In cell line experiments exposure to oestrogen results in a rapid increase in proliferation of ovarian cancer cells (33, 34). In addition, tamoxifen, an anti-oestrogen, has been reported to have beneficial effects in a few women with ovarian cancer (35).

1.2.3 Risk factors: Hereditary ovarian cancer

Although the lifetime risk of a woman developing ovarian cancer in developed countries is 1 in 70, a strong family history of either breast or ovarian cancer increases this risk substantially (36, 37). Hereditary ovarian cancer is uncommon, and accounts for 5-10% of all ovarian tumours. Although the factors mentioned above are thought to influence the risk of developing EOC in these women, germline mutations in the BRCA1 and BRCA2 breast/ovarian susceptibility genes overwhelmingly account for most hereditary cancers. In families with BRCA1 and BRCA2 mutations, the lifetime risk of developing ovarian cancer is 60% and 30% respectively (9, 38-40). However, the accuracy of this risk is debatable as the bias of most surgeons is to recommend bilateral oophorectomy in this population once child bearing is complete (9, 41). Although the risk is not completely negated with this procedure, it is considerably reduced (42). However, oophorectomy is not a reasonable option in young women wishing to retain their fertility. Epidemiological data from the Gilda Radner Familial Ovarian Cancer Registry show that oral contraceptive use is associated with a lower risk of developing ovarian cancer in BRCA1 and BRCA2 carriers (43). In conjunction with intensive screening, oral

contraceptives might be an attractive alternative in women who have not completed childbearing.

1.3   Pathology

Histology of the normal ovary is complex. During embryonic life a gonadal ridge is formed by a thickening of coelemic epithelium, which forms the serosal covering of the ovary. This epithelium, by a process of invagination, gives rise to the müllerian ducts from which the fallopian tubes, uterus and upper vagina form. The outer epithelial layer, or serosa, surrounds a dense stromal mass that contains arrested primary oocytes, developing primary and secondary follicles, a corpus luteum, and lymphatic and vascular drainage (8). All cellular subtypes are prone to cancerous transformation, and cancer of the ovary can be broadly categorised as epithelial, germ cell or stromal in origin. Ninety percent of all ovarian tumours are epithelial in nature, and consequent to common origins of the ovarian surface mesothelium and epithelium of the müllerian ducts, EOC can develop along 5 routes (8, 44). Serous cystadenocarcinomas are by far the most common, accounting for 60% of EOC's. They present as bilateral tumours in 50% of cases, and have a multiloculated, cystic appearance on gross pathology. They are composed of low columnar epithelial cells that morphologically resemble the epithelial cells of the fallopian tube mucosa. These cells lack intra-cellular mucin, and grow in a papillary histological pattern (44).

The remainder of EOC's consist of mucinous, endometrioid, clear cell and Brenner tumours. Mucinous cancers are composed of columnar cells that resemble endocervical epithelial cells, and contain abundant cytoplasmic mucin. These tumours comprise 10-20% of EOC's and present bilaterally in a quarter of cases. Endometrioid ovarian cancer, which account for 10-20% of EOC's, typically resembles endometrial tumours of the uterus. The corresponding cells are columnar with elongated dark nuclei, and grow in a tubular or cribriform pattern. Clear cell carcinomas are rare and comprise 3% of EOC's. They are composed of cells laden

with glycogen that are similar to glandular endometrial cells associated with pregnancy. Brenner tumours are composed of transitional cells, and are identical to tumours of the urinary tract. They account for less than 2% of EOC's and are typically unilateral on presentation (8, 44, 45).

The debate on precursor lesions resultant in invasive EOC, analogous to cervical intra-epithelial neoplasia progressing to invasive cancer (46), remains unresolved (10, 47, 48). Cellular atypia and metaplastic change on the surface epithelium of the ovary and of the epithelial lining of inclusion cysts adjacent to invasive neoplasia has been reported (49). However, it is rare to find these changes in the absence of invasive cancer (10). A further interesting observation is that cortical inclusion cysts are found in increased numbers in apparently normal ovaries contralateral to ovarian cancer, compared to ovaries from age-matched women without ovarian cancer (50). Whether this increased frequency represents a requirement for the development of EOC is questionable. There also remains intense debate about the nature of borderline tumours of Low Malignant Potential (LMP). Initially thought to be precursors of their invasive counterparts there is mounting evidence for their classification as a unique entity (51-55). Borderline tumours share similar histology patterns to their malignant counterparts, are able to metastasise in a transcoelemic fashion, but lack important invasive properties. Consequently, patient survival over 5 years is in the order of 95%, despite having apparently advanced disease (55). It is also rare to find foci of borderline tumours in otherwise frankly malignant tissue (10). In addition, recurrent borderline tumours are characteristically identical to that of the original lesion and do not appear to progress to invasive malignancies. There is also limited evidence of genetic dissimilarities between the 2 tumour subtypes. For example, p53 gene mutation and mutant protein overexpression is found in over 50% of invasive EOC, but rarely occurs in the borderline variant (52, 54).

Therefore, the histological classification of ovarian tumours reflects both the cell type and the biological behaviour of that tumour. The degree of cellular differentiation, nuclear atypia, and loss of architectural integrity, are summarised in a grading system that provides additional information on the behaviour of tumour

subtypes (8). In general, well differentiated tumours more closely resemble their normal counterparts, with poorly differentiated tumours at the other extreme. Defining the cellular origin and grade of the cancer is important, because of correlation with prognosis and occasional therapeutic implications. In addition, some pathologists report the mitotic activity index of the tumour, degree of DNA ploidy and percentage of surface epithelium involved in carcinomatous change, as these factors have shown to have some prognostic influence on disease outcome (56-58). However, these factors are weak prognosticators of clinical outcome and are generally not reported.

Invasive EOC metastasise in three ways. The earliest route of dissemination is transcoelemic, with exfoliated cells implanting along the surfaces of the peritoneal cavity. Spread via the lymphatic system, is mainly along the nodal chain in the pelvis, to the para-aortic region at the level of the renal vessels. These nodes are involved in 15 to 20 percent of stage I and II cases, and up to 50 percent in the latter stages. Haematogenous spread is rare and occurs late. The main sites of distant metastases are the liver and lungs, although spread to bone and brain is sometimes seen (7). The extent of metastatic spread is incorporated in the FIGO clinical staging process for this disease that is summarised in Table 1.2 and illustrated in Figure 1.2. This consensus standardisation by the International Federation of Gynaecologists and Obstetricians is vital, as it not only dictates the therapeutic options available to the clinician and patient, but is also an important prognostic factor in determining overall and progression-free survival of patients.

Table 1.2   International Federation of Gynaecology and Obstetrics (FIGO) staging system for ovarian cancer*

Stage I	Growth limited to the ovaries
Ia	Growth limited to one ovary, no malignant cells in ascitic fluid or peritoneal washing; No tumour on the external surface; capsule intact.
Ib	Growth limited to both ovaries, capsule intact, no malignant cells in ascitic fluid or peritoneal washings; No tumour on the external surface; capsule intact.
Ic	As with Ia or Ib but with tumour on the surface of one or both ovaries; or with capsule ruptured; or with malignant cells in ascitic fluid or positive peritoneal washings.
Stage II	Growth involving one or both ovaries with pelvic extension
IIa	Extension and/or metastases to the uterus and/or tubes. No malignant cells in ascitic fluid or peritoneal washings.
IIb	Extension to other pelvic tissues. No malignant cells in ascitic fluid or peritoneal washings
IIc	As in IIa or IIb but with tumour on the surface of one or both ovaries; or with capsule(s) ruptured; or with malignant cells in ascites or peritoneal washings
Stage III	Tumour involving one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal lymph nodes. Superficial liver metastases equals stage III. Tumour is limited to the true pelvis, but with histologically proven malignant extension to small bowel or omentum.
IIIa	Tumour grossly limited to true pelvis with negative nodes but histologically confirmed microscopic seeding of abdominal peritoneal surfaces
IIIb	Tumour of one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter. Nodes negative
IIIc	Abdominal implants > 2cm in greatest dimension and/or retroperitoneal or inguinal nodes
Stage IV	Growth involving one or both ovaries with distant metastases. If pleural effusion is present, their must positive cytologic test result to allot a case to stage IV. Parenchymal liver metastases equals stage IV.

*(From: Atlas of Tumour Pathology, 3rd series, eds. AFIP, Scully RE, Young RH, Clement PB.)

Figure 1.2 Schematic representation of FIGO clinical staging of epithelial ovarian cancer **(www.figo.org)**
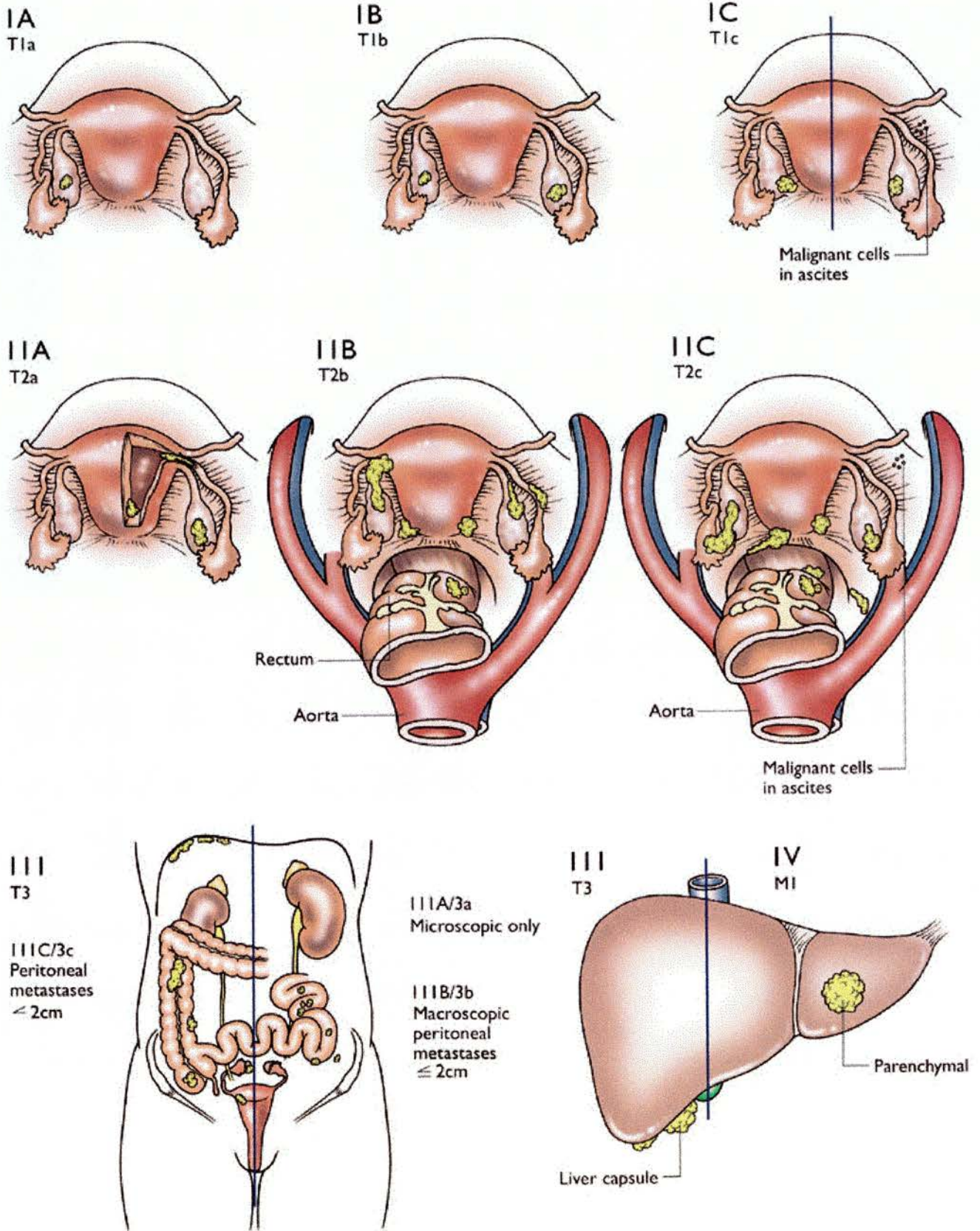
1.4 Clinical prognostic markers determining disease outcome of patients with ovarian cancer

"A prognostic indicator is a factor defined at a given time point which gives information on the subsequent clinical outcome (of a patient)", Eisenhauer, 1999 (59). Numerous clinicopathological variables have been evaluated that impact on the overall and disease free survival of patients with ovarian cancer (57-76). These include clinical stage; volume of post-operative residual disease; histological grade including mitotic index, DNA ploidy and cell type; presence of preoperative ascites; age and performance status of the patient and pre-treatment and intra-treatment levels of serum CA125. While most factors in various settings have been shown to be independent predictors of survival, the most important remain clinical stage and residual disease (60-67).

Clinical stage is determined at the time of primary surgery, using the FIGO staging system, which is summarised in Table 1.2. In general, patients with cancer confined to the ovary or pelvis, as in stages I and II, do considerably better than those in whom the cancer has metastasised beyond the pelvis. In most series, the prognostic influence of stage and substage remains significant even when other factors are considered in a multivariate assessment of disease outcome (62, 63). The role of maximal cytoreductive surgery in patients with ovarian cancer was first established in 1968 (68). Since then numerous studies have confirmed a better progression-free and overall survival of patients in whom there was minimal residual disease (RD) remaining after primary surgery (60, 61, 64, 66). The "minimal" now generally accepted is RD of less than 2cm as the maximal diameter of the largest remaining deposit of cancer. Adding weight to the argument for optimal debulking, is the conceptual theory that chemotherapy is more effectively delivered to smaller volume disease. Approximately 70% of patients present with advanced disease in whom survival is generally poor. The role of optimal debulking in this population is of vital importance. Even in those patients with hepatic metastases and/or malignant pleural effusions, Naik *et al.*, (2000) (66) and Munkarah *et al.*, (1997), (65) respectively, demonstrated that the volume of post-operative RD is an important determinant of

prognosis. More recently, Akahira and colleagues (2001) reported a survival advantage of 16 months in those patients with stage IV disease that were optimally debulked (69).

EOC, as mentioned previously, cluster into 5 different subtypes dependent upon their cell type. For any given stage, or volume of RD, clear cell carcinomas have the worst overall prognosis and endometrioid tumours have the best (8). In addition to their distinct morphologic appearance, there is increasing molecular evidence for their heterogeneity. In a recent publication, Ono *et al.*, (2000), used DNA microarray technology to examine the expression of 9121 genes in 4 serous and 5 mucinous ovarian cancers (70). Whilst they demonstrated differential up and down-regulation of 55 and 48 genes in all 9 cancer specimens versus normal ovary, they identified 115 genes that were differentially expressed between serous and mucinous tumours. This raises an interesting biological question of whether tumours of varying cellular subtypes should be grouped as a single entity for the purpose of molecular and clinical research, as it is frequently done in the case of EOC's.

The degree of tumour differentiation relates to the extent of cellular and nuclear atypia and architectural disruption of the organ. Most studies group the moderately and poorly differentiated tumours together as these have been shown to directly correlate with adverse prognosis in patients relative to those with well-differentiated cancers (71-73). In a similar vein, the importance of DNA ploidy (aneuploid versus diploid) in tumour specimens in predicting survival has also been investigated (71, 73-76). Only two studies appear to support its independent role in multivariate analysis when adjusted for other prognosticators (71, 75). However, this predictive role appears to co-segregate with early stage tumours and thus may be an important factor in distinguishing patients that have a better outcome in this population (75).

Intra-abdominal ascites is present in up to 40% of women diagnosed with ovarian cancer. Fluid accumulates as a result of lymphatic obstruction and increased secretion from the peritoneal surfaces. The ensuing electrolyte imbalance influences the patients' ability to tolerate treatment. The presence of pre-operative ascites has

been shown to be associated with poor survival in patients with ovarian cancer (60, 77-79). Puls *et al.*, (1996), reported a 5-year survival of 45% in patients with stage III disease without ascites, versus 5% survival in a comparative group with ascites (79).

The Performance Status (PS) is a measure of the severity of symptoms suffered by an individual as a consequence of their disease. In general these physical manifestations of the disease are quantified in a scoring system dependent upon which system is used. Several systems exist which include Kranofsky scales, WHO and GOG. The most convincing role of PS as a prognosticator of disease outcome was shown by Warwick *et al.*, (1995), in the long-term follow up of 362 patients diagnosed with advanced ovarian cancer between 1981 and 1991 in the West Midlands, UK (73). In their multivariate model, PS was the strongest predictor of survival above RD and serum albumin levels (73). Although other groups have been unable to attribute such importance to PS, their results generally support the independent prognostic value for this marker (80, 81). However, the issue of age *per se* as a prognostic indicator is difficult to evaluate. In a study of 12,316 women with ovarian cancer, Hightower *et al.*, (1994), reported that women over the age of 80 did considerably worse than younger patients (82). However, it was noted that those patients over the age of 80 underwent less aggressive surgery and were generally offered less cytotoxic chemotherapy. This did not reflect the performance status of the patient, but related more to the belief of the treating physician that older patients may not be able to tolerate the radical procedures required in order to effectively treat their ovarian cancer.

1.5   Clinical management of patients with ovarian cancer

Within the paradigm of cancer treatment, centralisation of services is crucial. A multidisciplinary approach to such management in specialist centres is mandate and preliminary results show improved survival of patients treated under such conditions (83, 84). It also provides the platform for patient recruitment in stringent clinical trials involving modified or new treatments.

1.5.1 Screening

In the overall management of ovarian cancer, screening for the detection of early disease must play a pivotal role. However, while significant inroads are being made in screening for hereditary ovarian cancer (9, 36, 85-87), effective tests in screening for sporadic tumours, that constitute over 90% of all ovarian cancers, remain elusive. Ideally, a screening test should have complete sensitivity to detect all positive cases, and sufficient specificity to rule out all negatives ones. In the past 15 years, an enormous amount of research has been dedicated to evaluating the role of pelvic ultrasonography and serum CA125 levels as potential screening tools for ovarian cancer (87-102).

In 1983, Bast and colleagues were the first to report the use of CA125 measurement in monitoring the course of patients with EOC (88). This large glycoprotein is secreted by the endothelial cells of most pelvic organs including normal ovary, and by mesothelial cells of pleura, pericardium and peritoneum. Levels are elevated in 1% of the normal population, 6% of patients with benign disease such as endometriosis and pleuritis, 28% of patients with non-gynaecological intra-abdominal malignancies, and 60% of patients with EOC (89, 90). CA125 serum levels are only elevated in 50% of patients with early stage EOC (91) and therefore the solitary use of CA125 as a screening tool for the early detection of EOC suffers from poor specificity and sensitivity. However, there is now considerable evidence to support the use of CA125 as a marker of response to therapy and as an indicator of

relapse during follow-up (92, 93). In 70% of patients a rising CA125 titre is a first indication of relapse, predating clinical relapse by a median of 4 months. While this may be useful in initiating treatment sooner, a clearer benefit of earlier detection in improving survival has not been clearly demonstrated (90). Moreover, the earlier introduction of chemotherapy must be weighed against the loss of treatment free time, anxiety and repeated hospital visits, as relapse is usually incurable. Nevertheless, CA125 measurement in predicting relapse has been universally adopted. Similarly, it is a useful marker in predicting progressive disease and treatment failure (90, 94). Adjuvant therapy can be tailored for such patients as to avoid the toxic side effects of treatment if unnecessary, thereby temporarily improving their quality of life.

With an improvement in radiological techniques in the past decade, the use of real-time ultrasonography and colour flow doppler in detecting early EOC has been evaluated (95-98). In the largest study to date, Jacobs et al., (1993) investigated the efficacy of regularly screening 22,000 postmenopausal volunteers using pelvic ultrasound in conjunction with serum CA125. Forty-one women underwent operations following abnormal results, in whom 11 cancers were discovered. There were altogether 19 ovarian cancers in the group of 22,000 women tested. The results were generally disappointing with the screening modalities having a positive predictive value of 20% and a poor sensitivity (91). These results are in concordance with other published literature (99-101). There are also on-going trials evaluating the role of CA125 as a primary test followed by ultrasound scanning as a secondary screen. Preliminary data is promising for this sequential strategy, showing some improvement in the overall sensitivity and specificity (102, 103). The role of several other tumour markers as screening tools for EOC such as CA19-9, CA15-3, MCA, PLAP and MOV-1 are currently being investigated (89, 90).

1.5.2 Surgery

The role of primary surgery can be broadly classified into 3 categories. The first aim is to make a definitive diagnosis of ovarian cancer. Once established, the clinical staging of this disease is performed by systematic examination of the pelvic organs, omentum, subdiaphragmatic areas, anterior abdominal wall, paracolic gutters, surface of the small and large bowel and palpation of pelvic and para-aortic lymph nodes (7). Accurate staging is of vital importance in planning further treatment for the patient. The third aim of primary surgery is therapy. Optimal cytoreductive surgery minimally involves total hysterectomy, bilateral salpingoopherectomy and omentectomy, with the aim of leaving less than 2 cm of macroscopic disease. Seventy percent of patients present with cancer that has spread beyond the ovaries and optimal cytoreduction is generally achieved in three-quarters of this population (7). As mentioned previously, the volume of post-operative residual disease is a powerful prognostic indicator of disease outcome, even in patients with extra-peritoneal metastases (60, 61, 65, 66). However, the value of more radical surgery involving pelvic and para-aortic lymphadenectomy in achieving optimal cytoreduction is controversial (104-111). Certainly Lymph Node (LN) status is included in FIGO staging (Table 2). Nevertheless, there is considerable evidence to suggest that widespread peritoneal involvement of the cancer (FIGO III & IV) is significantly more unfavourable than lymphatic invasion, and removal of diseased nodes does not appear to influence patient survival (106, 112, 113). However, the debate regarding LN resection in tumours confined to the ovaries (FIGO I) is not so clear. Most stage I tumours are removed by general gynaecologists and LN resection is not routinely performed, partly due to the lack of expertise for this complicated procedure. Numerous studies have reported 15-25% LN involvement in these apparently early stage tumours (105, 114), and patients with positive nodes have a significantly worse outcome than those with negative nodes (105, 106). Those with apparent stage I disease but positive nodes, would automatically be upstaged to IIIC under current FIGO classification and adjuvant therapies (not necessarily given in stage I disease) would need to be initiated. However, the 5-year survival in these patients with no disseminated peritoneal disease exceeds 60%, which is considerably

more than those with IIIC disease involving peritoneal metastases. Indeed there has been a proposal that perhaps the FIGO staging should be modified to separate patients with solely retro-peritoneal metastases from those with intra-peritoneal dissemination.

In addition to the radical procedure of lymphadenectomy, the issues concerning bowel and bladder resection with the aim of achieving optimal tumour debulking, have been reported but to a lesser extent. There is some support, for en-bloc removal of the recto-sigmoid segment with the tumour mass if densely adherent, but otherwise bowel resection should only be performed if eminent obstruction is suspected (115, 116).

Lastly, a conservative approach may be employed in a young woman wishing to preserve her fertility with well differentiated stage I disease. However, it is recommended that the remaining ovary be removed after child bearing is complete, as the future risk of developing ovarian cancer is unknown.

Although subsequent surgery is often employed to treat metastases, these procedures are predominately palliative.

1.5.3 Chemotherapy and Radiotherapy

Whole abdominal radiation therapy is rarely used in the treatment of ovarian cancer. The dose required to achieve an effective response is curtailed by the ensuing damage to kidneys and liver. Side effects of radiation to bowel and bladder are severe and generally unacceptable. However, radiation is occasionally useful for palliation to relieve pressure symptoms in the pelvis, vaginal bleeding and metastatic deposits in the brain and bone (7).

Chemotherapy has supplanted radiation therapy as the modality of treatment following primary surgery. Before 1980, the only drugs with significant efficacy in

treating ovarian cancer were the alkylating agents and doxorubicin (117). Platinum based compounds, such as cisplatinum and carboplatinum, were introduced into treatment scheduling in the mid-eighties (118), followed 10 years later by taxane based compounds. Over the past 5 years, clinical trials by the Gynaecologic Oncology Group based in the United States, and international collaborative trials by the European and Canadian consortiums have compared the use of cisplatinum/placitaxel regimes versus the traditional combination of cisplatinum/cyclophosphamide as primary adjuvant therapies in ovarian cancer (119-121). In patients receiving placitaxel in combination with a platinum-based compound, seventy-five percent attain some disease regression. However, the cumulative toxicity, especially neurotoxicity, curtails the use of these agents beyond 6 cycles (122). In those patients with minimal residual disease, 50% achieve a complete response as determined by second look surgery although, even in this population, a significant number of patients proceed to develop recurrent disease, with the overall time to recurrence being 16-18 months. Median survival is reported as approximately 38 months (120). In comparison only 60% of patients showed some response to cisplatinum /cyclophosphamide, with only 31% achieving a complete response. Median time to recurrence is 13 months and overall survival 24 months. Currently, the standardised regime in practice following surgical resection of EOC, is the use of placitaxel in conjunction with a platinum-based compound for 6 cycles.

However, 25-30% of EOC is resistant to drug manipulation and progression is rapid after primary surgery. In those patients with a partial or complete response after initial treatment, many develop recurrent disease that is generally resistant to further use of platinum based compounds. Placitaxel and topotecan are of some use in this latter population and are presently being evaluated in phase III trials (123-125).

1.5.4 Gene therapy

Gene therapy is defined as the treatment of disease at the level of the underlying genetic defect. It involves transferring nucleic acids into target cells for the purpose of perturbing or correcting some pathophysiological process. It was originally conceived and used in the treatment of inherited monogenetic defects such as adenosine deaminase deficiency and cystic fibrosis (126). Such a genetic strategy seems rational given the recognition that tumours typically develop in a progressive manner as demonstrated in familial adenomatous polyposis and colorectal cancer (127, 128). However, a progression model for the development of ovarian cancer has not been identified. Therefore it is unknown which genes are mutated in the early development of this cancer. Given the numerous alterations that may eventually result in tumour development and progression, there is no obvious single choice for a therapeutic gene. Although expertise in this field is rapidly expanding the search for an ideal target vector for gene delivery presents another challenging obstacle (126, 129). Despite this, a phase 1 international clinical trial is underway to evaluate whether an adenoviral vector containing wild type p53 given intraperitoneal in conjunction with traditional adjuvant chemotherapy, will improve survival of patients with ovarian cancer and increase chemotherapy efficacy. This appears to be premature given the controversial results of mutated p53 overexpression correlating with patient survival and progression-free interval and the present system for gene delivery. Other genes being subjected to clinical trials in breast and ovarian cancer include Her-2/neu and BRCA1 (130, 131).

1.6 Conclusion

Ovarian cancer is a common disease in western societies with a poor clinical outcome. Advances in the surgical and chemotherapeutic management has significantly improved the quality of life of patients suffering with this disease, but have yet to make a dramatic difference in determining the clinical outcome of these patients. Although several clinical and pathological markers of disease outcome in ovarian cancer have been identified, there is a need for molecular markers that could reliably predict the overall survival and progression-free interval of patients. In addition, a better understanding of tumour biology may lead to new and more effective therapies in treating ovarian cancer.

Chapter Two   Molecular genetics of ovarian cancer

2.1   Introduction

Cancer development and progression is thought to result from an accumulation of mutations in multiple genes that are important for normal cellular function, that ultimately leads to uncontrolled proliferation and tumour growth (127). Subsequent mutations may select for more aggressive events such as invasion into surrounding tissue and metastases. Aberrant expression of oncogenes stimulates this process and tumour suppressor genes inhibit it. Abnormal activation of oncogenes and inactivation of tumour suppressor genes have been identified as essential steps during carcinogenesis. Over 100 putative proto-oncogenes and tumour suppressor genes have been described although only a few have been studied in ovarian cancer (132). The list is likely to grow rapidly with the utilisation of DNA microarray technology to identify novel genes involved in ovarian tumourigenesis. A summary of genetic mutations identified in ovarian cancer is presented in Table 2.1.

Table 2.1   Common genetic mutations identified in ovarian cancer

χ (Chromosome)	Ref.	Mutation	Genes implicated to be involved in ovarian cancer
3	(148, 149)	Deletion 3p21.1-22 3p LOH – 3p12-13; 3p14.2 3p23-24.4; 3p24-25	*AKT* *FRA3B*
5	(150, 151)	5q LOH 5q33.3-5q34	*APC* Lynch syndrome II *c-FMS* 50-75% over expression in EOC
6		6q LOH	
7		Deletion 7q31.1-31.3	Putative TSG – yet to be identified
8	(152)	8q24	*c-MYC* 30% over expression/amplification
9	(153)	LOH 9p21, 9q31, 9q32-34	$p16^{Ink4a}$ Mutations rare $p15^{Ink4b}$ $p14^{ARF}$
11	(154-156)	Deletion 11p13 & 11p15.5 Deletion or Translocation of 11q13 Deletion 11q23.3	*H*-Ras *CCND1* Amplification/mutation rare
12	(157)	12p13	$p27^{Kip1}$ 40% EOC loss of expression (CDKI) *K-RAS* 5-30% altered in EOC.
13	(147, 158)	Mutation at 13q12-13 LOH 13q14	*BRCA2* *Rb* LOH 30-40% in EOC. However LOH does not coincide with loss of *Rb* expression
17	(142, 159-164)	Allele loss at 17p13.1-13.3 LOH 17q21	*OVCA1* & *OVCA2* function unknown *p53* multiple mutations (missense predominately) *BRCA1* Familial tumours HER-2/*neu* 10-40% over expression/amplification
18	(165)	18q LOH	Advanced stage, locus near *DCC* gene
X	(166)	Loss of X	Possible early event in tumour development

The genes relating to cell cycle regulation are discussed in detail in subsequent sections. However, some of the other genes that have been studied extensively in ovarian cancer warrant further mention.

HER-2/*neu* (*erbB-2*) is located on chromosome 17q21-22 and encodes a 185 Kda transmembrane glycoprotein with tyrosine kinase activity. The phosphorylation cascade mediated by this oncoprotein promotes cell proliferation and differentiation through activation of the RAS-GTP signalling pathway (133). Gene amplification and/or protein overexpression has been observed in several types of tumours (134-136). In ovarian cancer, HER-2/neu overexpression occurs in approximately 30% of cases and correlates with poor survival. One possible explanation for this finding, is that HER-2/neu overexpression may result in cellular resistance to platinum-based chemotherapy regimes (137, 138). A recent clinical trial in metastatic breast cancer patients, found a significant improvement in disease response rates to cisplatinum when combined with an anti-HER-2/neu antibody (Herceptin) (139). Anti-HER-2/neu antibody also appears to mediate an increased sensitivity to cisplatinum in chemoresistant ovarian cancer cell lines containing multiple copies of HER-2/*neu* (140). However, a recent phase I trial utilising an anti-erbB-2 single-chain antibody-encoding adenovirus in patients with advanced ovarian cancer, failed to show any clinical benefit despite no obvious dose-limiting, vector-related toxicity (141).

The *BRCA1* gene is located on chromosome 17q21 and encodes a large 220 kDA protein expressed most abundantly in testis, breast and ovarian tissue (142). *BRCA1* is thought to function as a tumour suppressor gene, as the normal copy of *BRCA1* is invariably deleted in ovarian cancers that arise in women who inherit a mutant *BRCA1* gene. In addition, the introduction of wild-type *BRCA1* has been shown to inhibit growth of some breast and ovarian cancer cell lines (143). However, the exact cellular functions and interactions of the protein product remain unknown. Chromosomal mutations have been reported in approximately 50% of hereditary ovarian cancer families and carriers of these germ-line mutations have a 30-80% life-time risk of developing ovarian or breast cancer (144). Loss of heterozygosity at the *BRCA1* locus occurs frequently in sporadic ovarian cancer, although somatic

mutations of the remaining copy are rarely observed. Preclinical studies have demonstrated that intraperitoneal injection of retroviral vectors expressing BRCA1*sv*, a normal splice variant of BRCA1, can inhibit the growth of established intraperitoneal tumours in a nude mouse xenograft model (145). An initial phase 1 clinical study using intraperitoneal infusions of BRCA1*sv* in patients with advanced ovarian cancer, reported promising results relating to vector stability, minimal toxicity and the absence of an anti-BRCA1 antibody response (130). However, a subsequent phase II trial by the same group was prematurely terminated due to conflicting results of vector stability, the rapid development of anti-BRCA1 antibodies and importantly, no disease stabilisation (146). A second breast/ovarian cancer susceptibility gene (BRCA2) has been identified that maps to chromosome 13q12 (147). Ovarian cancer has been reported to occur in 10-35% of carriers with hereditary *BRCA2* mutations (133). Similar to *BRCA1*, loss of heterozygosity at the *BRCA2* locus is frequently observed in sporadic ovarian cancer, but somatic mutations remain rare (147).

Methylation-induced silencing of the *BRCA1* promoter with consequent reduction in BRCA1 mRNA has been demonstrated in 15% of sporadic breast and ovarian cancers. This does not appear to be the case in other solid tumours and indirectly may provide evidence for its pathological significance. In contrast, the *BRCA2* promoter has been shown to have few or no methylated dinucleotides in sporadic ovarian tumour DNA when compared with non-tumour DNA. Indeed in these cases BRCA2 mRNA is significantly elevated. It is also possible that downstream BRCA-interacting proteins may be affected by other events producing carcinogenic sequalae similar to those resulting from BRCA dysfunction. As previously mentioned the role of role of BRCA is largely unknown and with an increasing understanding may be shown to play a significant role in the development of sporadic tumours.

2.2 Cell cycle regulation

Regulation of cellular proliferation by external mitogens is governed through receptor mediated signalling pathways, which ultimately converge on cell cycle genes. The aberrant expression of cell cycle genes, resulting in cellular disruption, has been implicated in the development of most human cancers. In order for cells to replicate, they must pass through four distinct phases G_1, S (DNA synthesis), G_2 and M (Mitosis). Progress through these phases is regulated by molecular checkpoints to ensure DNA and cellular integrity prior to cell division (167). The orderly transition of cells through G_1 is the most critical step in this process. It is the restriction point in mid to late G_1 that determines whether a cell progresses to cell division, cell cycle arrest, cellular differentiation or apoptotic cell death. Progress to this point in G_1 requires a balance between positive and negative regulatory factors. The interaction of these factors (cyclins, cyclin dependent kinases (CDK's) and cyclin dependent kinase inhibitors (CDKI's)) is crucial for the eventual phosphorylation of pRb (168-171). Phosphorylation inactivates pRb, which results in the release of the E2F family of transcription factors that in turn activate a set of target genes that are essential for entry into S phase (172, 173). There are two important pathways responsible for the phosphorylation hence inactivation of pRb, which include cyclin D1/CDK4-6/p16^{Ink4a} and cyclin E/CDK2/p27^{Kip1}. Both these pathways are influenced by the CDKI p21$^{Waf1/Cip1}$, the levels of which are regulated by p53. A schematic diagram illustrating the events that lead to the phosphorylation of pRb is shown in Figure 2.1.
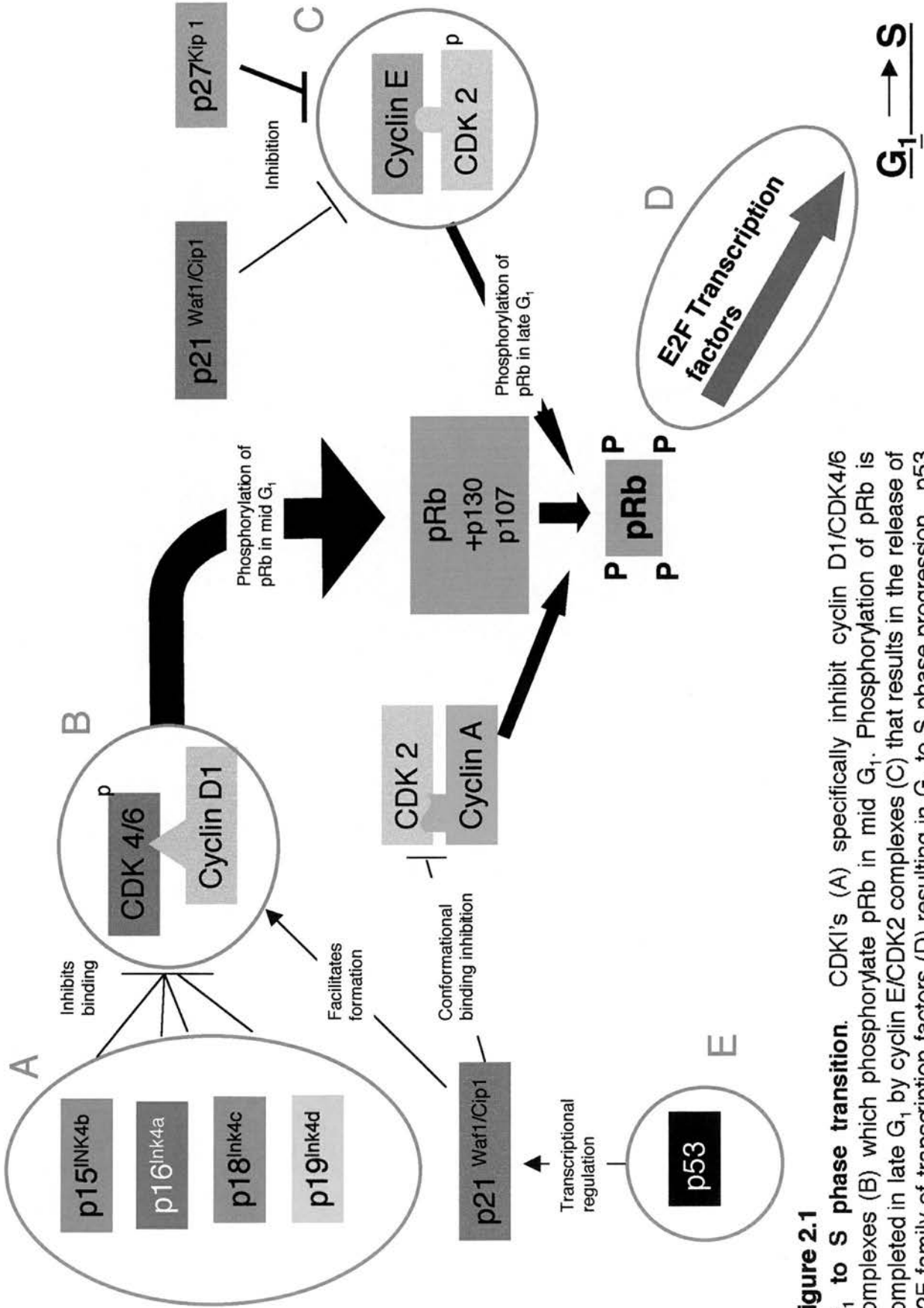
Figure 2.1

G₁ to S phase transition. CDKI's (A) specifically inhibit cyclin D1/CDK4/6 complexes (B) which phosphorylate pRb in mid G₁. Phosphorylation of pRb is completed in late G₁ by cyclin E/CDK2 complexes (C) that results in the release of E2F family of transcription factors (D) resulting in G₁ to S phase progression. p53 (E) exerts its effect on the cell cycle by activating transcription of the CDKI p21^Waf1/Cip1 which facilitates formation of cyclin D1/CDK4 but inhibits cyclin E/CDK2. The main inhibitor of cyclin E/CDK2 is the CDKI, p27^Kip1.

The retinoblastoma gene was the first well-defined tumour suppressor gene. Familial retinoblastoma is a disease in which children develop retinal tumours that often affect both eyes (174). Allelic loss at the *Rb* locus was found in such individuals, with mutations demonstrated in the remaining copy (175, 176). Its role as a tumour suppressor gene was confirmed by experiments on *Rb* knockout mice. Mice homozygous for *Rb* mutations died in-utero because of defects in haematopoiesis and neuronal cell death, and although heterozygote (*Rb* +/-) mice were viable, many spontaneously developed thyroid and pituitary tumours (178). Subsequent experiments in those mice developing tumours revealed loss of the remaining wild type *Rb* allele (177, 178).

pRb is a 928 amino acid nuclear phosphoprotein encoded by a gene on chromosome 13q14 (179). *Rb* gene mutations have been described in a wide variety of human neoplasms including gall bladder, breast and lung cancers (180-182). Loss of heterozygosity at the *Rb* locus is a reported frequent event in epithelial ovarian cancers, but is rarely associated with altered pRb expression (183, 184).

The putative role of cyclins as oncogenes has strengthened in the past few years. They are pivotal in binding to, and directing CDK's (185) to appropriate substrates, and phosphorylating them during specific phases of the cell cycle. Regulators of G_1 progression in mammalian cells include D-type cyclins (D1, D2, D3) and cyclin E (169). D-type cyclins associate with CDK4 and CDK6 initiating pRb phosphorylation in mid-G_1 (173, 186), while cyclin E binds to CDK2 and completes phosphorylation of pRb later in the G_1 phase (187). The most strongly implicated of the D-type cyclins, in neoplastic transformation of cells is cyclin D1 (188). Experiments show that when cyclin D1 is overexpressed in fibroblasts, pRb is phosphorylated earlier and progression through G_1 is accelerated (189). Conversely, when anti-cyclin D1 antibodies are injected in early to mid-G_1 phase, cell cycle arrest is observed. The gene encoding cyclin D1 is located on chromosome 11q13 (*CCND1*). Dysregulation of *CCND1* can occur through several mechanisms. In parathyroid adenomas an inversion involving 11q13 and 11q15 results in *CCND1* coming under the control of the parathyroid gene promoter (190, 191). In B cell

lymphomas a reciprocal chromosomal translocation occurs at the *Bcl1* breakpoint and expression of *CCND1* is regulated by the immunoglobulin heavy chain enhancer (192, 193). However, amplification of *CCND1* is the most common route of cyclin D1 overexpression in many solid tumours including head and neck, breast and pancreas (194-197).

Cyclin E accumulates in late G_1, and is downregulated in S phase (198). Transcription of *cyclin E* is activated by the initial phosphorylation of pRb. E2F transcription factor complexes negatively regulates the *cyclin E* gene promoter, and phosphorylation of pRb results in the release of this inhibitory mechanism (199). Regulation of cyclin E protein levels is not fully understood. Gene amplification has been observed in a small proportion of breast and colorectal tumours with abnormal protein expression found to correlate with advanced stage and poor grade in breast carcinomas, suggesting its potential role as a prognostic marker (195, 200).

The activities of cyclin-CDK complexes are regulated by CDKI's. CDKI proteins are grouped into two categories based on their protein sequence homologies and putative CDK targets. The first family include the Cip/Kip proteins whose actions broadly affect the activity of cyclin D-, E- and A-dependent kinases. The second class includes the Ink4 proteins that specifically inhibit the catalytic subunits of CDK4 and CDK6 (171).

A conserved amino-terminal domain, but highly divergent carboxyl-terminals define the structural and functional diversity of the Cip/Kip family members that include p21$^{Waf1/Cip1}$, p27^{Kip1} and p57^{Kip2}. The gene encoding p21$^{Waf1/Cip1}$ is located on chromosome 6p21.2 and contains 3 exons of 68, 450 and 1600 base pairs (201). The amino terminus of p21$^{Waf1/Cip1}$ protein binds cyclin-CDK complexes whereas the carboxyl end contains PCNA binding and inhibitory activity (202). p21$^{Waf1/Cip1}$ is involved in inducing growth arrest, terminal differentiation and apoptosis of cells (203). Although, gene mutations are rarely present in human cancers (204, 205) loss of protein expression is frequently observed (206-208).

p21$^{Wafl/Cip1}$ protein levels are predominately regulated at the transcriptional level (203). Wild type p53 induces *p21$^{Wafl/Cip1}$* transcription in response to DNA damage through direct interaction with p53-binding consensus sequences in the *p21$^{Wafl/Cip1}$* promoter region, thus exerting its growth suppressive effects (209). Initially thought to be a surrogate marker of p53 function, there is now considerable evidence that p21$^{Wafl/Cip1}$ can exert inhibitory effects on the cell cycle and induce apoptosis independent of p53 expression. Studies using fibroblasts from *p53* knockout mice, breast cancer cells with known *p53* mutations and TGFβ activation of ovarian cancer cell lines lacking functional p53 have demonstrated upregulation of p21$^{Wafl/Cip1}$ independent of p53-induction (210-212).

p27^{Kip1} is another member of the Cip/Kip family. A gene located at chromosome 12p12-12p13.1 encodes p27^{Kip1}. Point mutations and homozygous deletions are rare events in the development of human cancers (157), and levels of p27^{Kip1} protein are predominately regulated at the posttranscriptional level through degradation by the ubiquitin pathway (213). p27^{Kip1} levels are high in quiescent cells and decrease during G1 phase reaching the lowest point in S phase. This differential decrease is due to a shorter half-life of the protein in G$_1$ as a result of a 6-8 fold increase in degradation (171).

The inhibitory mechanism of p27^{Kip1} is not fully understood. In G$_1$ arrested cells, p27^{Kip1} is preferentially bound to cyclin E/CDK2, although during proliferation it appears to be associated with cyclin D1/CDK and cyclin A/CDK complexes (214). It is this sequestration of p27^{Kip1} from cyclin E/CDK2 to cyclin D1/CDK complexes that appears to favour progression into S phase. Transgenic *p27^{Kip1}* knockout mice display features of multi-organ hyperplasia, hypertrophy and tumourigenesis (215-217). In addition to CDK inhibition, there is also evidence to suggest that overexpression of p27^{Kip1} induces apoptosis in different cancer cell lines (218) and levels may also relate to the degree of differentiation of the tumour type (171). Decreased expression or loss of p27^{Kip1} has been shown to correlate with poor prognosis in patients with breast, prostate, colorectal and gastric cancers (219-222).

Ink4, the second category of CDKI's includes p15^{Ink4b}, p16^{Ink4a}, p18^{Ink4c} and p19^{Ink4d}. p16^{Ink4a} is the most comprehensively studied within this group (223). p16^{Ink4a} specifically targets CDK4 and prevents the association of CDK4 with cyclin D1 thereby inhibiting phosphorylation of important downstream targets (224). Somatic mutations, homozygous deletions or DNA methylation of the *p16^{Ink4a}* gene located on chromosome 9p21, have been detected in a high percentage of human cancer cell lines and some primary tumour types (225, 226). Levels of p16^{Ink4a} are predominately regulated by the ubiquitin-proteosome pathway. Other observations suggest that a bi-directional feedback loop may exist between pRb and p16^{Ink4a}. Li *et al.*, (1994), (227) demonstrated that transcription of *p16^{Ink4a}* is suppressed by pRb in cultured cells, and Fang *et al.*, (1998), (228) showed that *pRb* is transcriptionally repressed by p16^{Ink4a}. Consistent with these findings, *p16^{Ink4a}* gene deletion and *pRb* deficiency are reported to be inversely correlated in some tumour types (180). In addition, an association between altered cyclin D1 and pRb in oesophageal tumours has also been reported (229).

The cell cycle regulatory effects of the nuclear phosphoprotein p53 are also manifest in the G1 phase. Wild type (wt) p53 nuclear phosphoprotein, consisting of 393 amino acids, is the product of a tumour suppressor gene located in the short arm of chromosome 17. This modular protein has several functional domains (230). The amino-terminal region binds to a target DNA sequence, promoting transcription and thereby activating the expression of particular genes. These include genes involved in cell cycle arrest (e.g. p21, GADD45, *mdm*-2) (231) and those modulating apoptosis (e.g. BAX, Bcl-2) (232). The carboxyl-terminal region is responsible for the formation of stable tetramers, the form in which wt p53 is found. Adjacent to this, there is a region involved in the recognition and repair of damaged DNA. Under normal conditions wt p53 is expressed at low levels, does not interfere with routine cellular functions, and has a short half-life of only a few minutes (233). However, following a stress signal such as hypoxia or DNA damage, there is a rapid increase in levels of wt p53, and subsequent phosphorylation and acetylation results in its activation as a transcription factor. Consequently, it can induce cell cycle arrest, DNA repair, or apoptotic cell death.

Inactivation of this tumour suppressor gene is implicated in the development of more than 50% of all human cancers (162). Common abnormalities include loss of heterozygosity (LOH), missense mutations, re-arrangements, frame shift mutations and deletions. The majority of mutations in ovarian cancer are missense mutations that are present in the highly conserved domains involving exons 5 to 8 (161, 234). The resultant abnormal p53 protein has the ability to hetero-dimerize with wt p53 rendering it inactive. Moreover, some mutated p53 proteins can enhance transformation and progression of cells when injected into wt p53 nullizygous cells, suggesting properties other than hetero-dimerization with wt p53 contribute to cellular alterations (162, 235). Mutated p53 protein is generally resistant to degradation and has a prolonged half-life, which allows its detection by immunohistochemical staining (161, 236, 237). These properties offer a simple approach for studying the role of *p53* gene alterations in clinical material but assumes concordance between *p53* mutations and positive protein staining. Several studies have demonstrated a significant correlation between *p53* mutations and aberrant protein expression, especially for missense mutations that are present in a high percentage of ovarian cancers (238). The role of truncating mutations resulting in no protein expression is discussed in chapter 5. *p53* mutation and/or protein overexpression is predictive of poor clinical outcome of patients with many different types of cancers (208, 239, 240).

In conclusion, progress through the cell cycle, in particular G1 to S phase transition is regulated by complex interactions between positive and negative regulators that ultimately determine whether the cell is committed to division, growth arrest, differentiation or apoptosis. Section 2.3 examines the published evidence for deregulation of specific cell cycle genes in epithelial ovarian cancer.

2.3 Aberrant expression of specific cell cycle genes in epithelial ovarian cancer

2.3.1 p53

Gene mutations and/or overexpression of p53 have been detected in 30-80% of epithelial ovarian tumours (234, 241-243). However the prognostic role of aberrant p53 expression in determining the outcome of patients with epithelial ovarian tumours is far from conclusive. Several studies have identified p53 overexpression as an adverse prognostic factor in EOC (234, 241-245), although only two have shown it to be independently prognostic when analysed with clinicopathological determinants of disease outcome (242, 243). In contrast, there are a number of studies that suggest expression of p53 has no prognostic value in EOC (246-249). Interestingly, the data presented by Antilla (242), Eltabbakh (241) and Klemi (243), demonstrate that p53 overexpression co-segregates significantly with tumours of serous origin. Epithelial ovarian tumours constitute several subtypes distinguishable on histology and have subtle biological and behavioural differences. For example clear cell carcinoma is more aggressive than other types of epithelial ovarian tumours (8). Hence, a major reason for contradictory findings in the published literature may be the variant nature of the tumour cohort. Further controversies exist regarding the role of p53 expression in chemoresistant ovarian tumours. In approximately 20% of patients, EOC is refractory to chemotherapeutic intervention. These agents commonly promote tumour regression by inducing apoptosis, a process in which p53 has a crucial role. Marx (1998) (250) and Buttitta (1997) (251) both found a significant relationship between p53 overexpression and chemoresistant disease, in contrast to findings published by Van der Zee (1995) (252) and Renninson (1994) (253). A summary of published data on p53 alteration and clinical outcome in EOC is shown in Table 2.2. The studies were identified by a systematic search on Medline using the key words, ovarian cancer and p53. Studies not related to survival and/or correlation to clinicopathological features of ovarian cancer are not included in this table.

Table 2.2   Literature review of p53 alteration and clinical outcome in ovarian cancer.

Authors	Total	EOC	Tumour number	Overall survival		Progression-free interval		Findings
				Univariate	Multivariate	Univariate	Multivariate	
Levessque et al., (2000) (245)	120	S M E U CC Mix	47 8 20 21 18 6	<0.01	NS	0.04 (5-year PFI)	NS	Immunoassay study. Associated with stage, grade, RD and failure to respond to chemotherapy.
Antilla et al., (1999) (244)	305	All subtypes. Different numbers used for each analysis		<0.0005 (5-year survival)	NS	0.0006 (5-year PFI)	0.01	Associated with grade, stage, RD and serous EOC. Expression combined with p21 $^{Waf1/Cip1}$ discussed in section 2.3.2
Werness et al., (1999) (249)	85	S M E CC Misc.	46 10 10 8 9	NS	NS	0.01	NS	Associated with serous EOC, grade and stage.
Wen et al., (1999) (234)	105	S M E U CC B Mix	78 3 11 1 4 1 7	0.049 (expression alone) 0.046 (mutation alone) 0.02 (combination)	NS			Significant association between gene mutation and protein expression. p53 expression associated with grade.
Silvestrini et al., (1997) (248)	168	S M E U Misc.	102 31 8 14 13	NS		NS		No association with clinical and pathological parameters.
Eitabbahh et al., (1997) (241)	221	S Misc.	126 95	0.0018	NS			No association with other clinical parameters.
Herod et al., (1996) (247)	70	S M E CC Misc.	50 3 10 6 1	NS	0.05			Cell line data suggests mutant p53 confers cisplatinum resistance.
Klemi et al., (1995) (243)	136	S M E CC Misc.	75 8 34 17 2	0.002	0.008			Associated with histological subtype and grade.

Authors	Total	EOC	Tumour number	Overall survival		Progression-free Interval		Findings
				Univariate	Multivariate	Univariate	Multivariate	
Levesque *et al.,* **(1995)** (254)	90	S M E CC U Misc.	36 8 21 7 10 8	0.06	NS	0.03	NS	Associated with serous subtype, grade, stage and RD. Immunoassay study.
Marks *et al.,* **(1991)** (246)	107	All subtypes. Numbers not reported		NS				69 samples from primary laparotomy, 38 from second look surgery.

S: Serous
M: Mucinous
E: Endometrioid
CC: Clear Cell
B: Brenner
U: Undifferentiated
Misc: Miscellaneous
Mix: Mixed Epithelial Cancer
NS: Not significant
EOC: Epithelial Ovarian Cancer

2.3.2 Cyclin Dependent Kinase Inhibitors: p21^Waf1/Cip1, p27^Kip1, p16^Ink4a

The role of altered p21$^{\text{Waf1/Cip1}}$ expression as a prognostic biomarker has been investigated in a range of human cancers.   Loss of p21$^{\text{Waf1/Cip1}}$ expression is significantly associated with reduced survival of patients with bladder, oesophageal and oral cancer (206-208).   However, its prognostic role in ovarian cancer is not clear.   While several studies have examined the expression of p21$^{\text{Waf1/Cip1}}$ in EOC (244, 245, 249, 255-257), only 2 have reported a significant association with clinical outcome (244, 255).   However, both these studies found p21$^{\text{Waf1/Cip1}}$ was not an independent prognostic factor when stratified with clinicopathological determinants of disease outcome in multivariate analysis.   One other study warrants mention. Costa *et al.*, (1999), demonstrated an independent prognostic role of p21$^{\text{Waf1/Cip1}}$ expression and disease outcome in epithelial tumours, although the value of this result is questionable.   The cohort contained cancers of peritoneal and ovarian origin, different histological subtypes and was inclusive of borderline tumours for survival analyses (258).

Expression of p21$^{\text{Waf1/Cip1}}$ is predominately regulated by wt p53 although as mentioned previously, p53-independent mechanisms of induction are likely to exist. In some studies of ovarian cancer, failure to demonstrate a correlation between p21$^{\text{Waf1/Cip1}}$ expression and p53 alteration supports a p53-independent mechanism of induction (245, 255).   In addition, although Anttila *et al.*, (1999), demonstrated a significant co-segregation between these 2 molecules in a large series of ovarian tumours, they reported increased power in predicting clinical outcome when combining the abnormal expression of p53 and p21$^{\text{Waf1/Cip1}}$ than either alone (244). Several other authors (234, 238, 244) have also supported this finding.   A summary of the published data on p21$^{\text{Waf1/Cip1}}$ expression and clinical outcome in ovarian cancer is shown in Table 2.3.

Table 2.3   Literature review of aberrant p21$^{Waf1/Cip1}$ expression and clinical outcome in ovarian cancer.

Authors	Total	EOC	Tumour number	Overall survival		Progression-free survival		Findings
				Univariate	Multivariate	Univariate	Multivariate	
Schmider et al., (2000) (255)	106	S M E U CC B Mix	52 8 21 13 9 2 1	0.033	NS	NS		No association with p53 expression. Levels related to FIGO stage, and RD. Significantly reduced survival in univariate analysis in tumours with p53 overexpression with concurrent loss of p21$^{Waf1/Cip1}$.
Levessque et al., (2000) (245)	120	S M E U CC Mix	47 8 20 21 18 6	NS		NS		No association with p53 expression or any pathological or clinical variables. Trend towards reduced survival in p53 positive /p21$^{Waf1/Cip1}$ negative tumours.
Baekelandt et al., (1999) (257)	185	S Misc.	138 47	NS		NS		p21$^{Waf1/Cip1}$ expression associated with stage and p27^{Kip1} expression. No association with p53 status.
Anttila et al., (1999) (244)	305	S M U CC Misc.	111 36 84 32 53	0.012	NS	NS		Expression associated with subtype, grade, stage, RD and p53 expression. Independent prognostic significance of p53 positive / p21$^{Waf1/Cip1}$ negative tumours.
Costa et al., (1999) (258)	117 SEN's	S M E CC TCC smcc	74 16 8 7 10 2	$<10^{-6}$	$<10^{-6}$			Study included peritoneal and ovarian tumours. Also included were borderline cancers. Levels associated with p53 expression
Werness et al., (1999) (249)	85	S M E CC Misc.	46 10 10 8 9	NS		NS		No association with clinicopathological features. Significantly reduced survival in p53 positive / p21$^{Waf1/Cip1}$ negative tumours on univariate analysis.

SEN's: Surface epithelial neoplasms (including primary peritoneal and ovarian tumours)

S:	Serous	U:	Undifferentiated	NS:	Not significant
M:	Mucinous	Misc:	Miscellaneous	EOC:	Epithelial Ovarian
E:	Endometrioid	Mix:	Mixed Epithelial Cancer		Cancer
CC:	Clear Cell	TCC:	Transitional cell carcinoma		
smcc:	Small cell carcinoma				

Decreased expression or loss of p27^{Kip1} correlates with poor prognosis in patients with breast, prostate, colorectal and gastric cancers (219-222). The literature pertaining to expression in ovarian cancer is limited. Newcombe *et al.*, (1998), were first to report an independent survival advantage beyond 5 years in patients with ovarian tumours demonstrating high p27^{Kip1} levels. The multivariate analysis was modelled against accepted clinical prognostic parameters of stage, grade, performance status and residual disease (259). However, a subsequent larger study by Baekeland *et al.*, (1999), incorporating 185 patients failed to confirm this result (257).

Deletions and point mutations in the *p16^{Ink4a}* gene are common in a variety of tumours including oesophageal cancers, squamous cell carcinomas of the head and neck and glioblastomas (229, 260-262). Loss of heterozygosity of *p16^{Ink4a}* occurs in 30 – 40% of ovarian tumours although mutations of the remaining allele are rare, which raises the possibility that hypermethylation of the *p16^{Ink4a}* gene promoter may result in decreased transcription of this gene. However, in serous ovarian tumours there is no evidence to suggest that methylation of the *p16^{Ink4a}* gene occurs (153, 263). Posttranscriptional modification or enhanced degradation may also be responsible for altered expression of p16^{Ink4a} (153). Aberrant expression of p16^{Ink4a} has not been shown to be prognostic in epithelial ovarian tumours (153, 263).

2.3.3 Cyclins: Cyclin D1, cyclin E

Cyclin D1 overexpression with or without gene amplification has been identified as an adverse prognostic biomarker in lung (264), pancreas (265), and tongue carcinoma (261). Gene amplification or rearrangement is rare in ovarian cancer (155), although increased levels of mRNA and overexpression of protein has been reported in 14%-95% of invasive tumours (155, 266, 267). External mitogenic signalling and regulation by other molecules integral to G1/S phase progression may account for this over expression.

The literature pertaining to cyclin D1 expression and clinical outcome in ovarian cancer is limited, and only one study has demonstrated a prognostic role for this protein in EOC. Barbierra *et al.*, (1999), reported a significant relationship between cyclin D1 overexpression and shorter progression-free interval in a small series of 24 patients with invasive ovarian malignancy (267). This subgroup of patients was derived from a larger study in which the authors were investigating cyclin D1 expression at the mRNA and protein level in benign, borderline and invasive ovarian tumours. The authors also found higher cyclin D1 levels in serous EOC and advanced stage disease. In contrast to this latter association with advanced features of malignancy, three studies have reported significant overexpression of cyclin D1 in borderline and well differentiated tumours (155, 266, 268). The reason for this is unclear and the authors postulate that deregulation of cyclin D1 may be an early event in the development of ovarian cancer. A further explanation cited is that many borderline and some low grade tumours may be distinctly different lesions and have pathogenesis different from that of higher grade cancers. Other studies have reported no association between protein expression and features of malignancy (269, 270). The role of cyclin D1 in the pathogenesis of ovarian cancer is controversial and larger studies are required to determine its relationship to features of malignancy and clinical outcome. A summary of the published data on cyclin D1 expression and clinical outcome in ovarian cancer is shown in Table 2.4.

Table 2.4 Literature review of cyclin D1 expression and clinical outcome in ovarian tumours

Authors	No.	Type	EOC Type/no.		Comment	Clinical outcome
Barbieri et al., (1999) (145)	104	55 benign 12 LMP 37 EOC (32 primary) (5 recurrence)	S M E Oth	16 6 21 12	mRNA (RT-PCR) and protein(western blot) study. Significant increase in mRNA levels from benign to borderline to invasive tumours.	24 patients with invasive EOC evaluable for clinical outcome. Significantly shorter PFI in those with cyclin D1 overexpression on univariate analysis. Not significant with DSS.
Sui et al., (1999) (257)	79	22 benign 23 LMP 34 EOC (all subtypes of EOC, numbers not specified)			Immunohistochemistry study. Significant reduction of cyclin D1 and p27^{KIP1} expression in benign to borderline to invasive tumours. (In EOC, increased levels of cyclin D1 expression in grade 1 cancers compared to grade 2 and 3.	Not investigated.
Dhar et al., (1999) (260)	81	81 tumours (inc. LMP) (not specified)	S M E CC Mix	37 13 14 11 6	Immunohistochemistry study. No association with tumour grade or clinical stage.	NS (PFI) / NS (DSS)
Mascuillo et al., (1999) (144)	64	64 EOC (56 primary) (9 recurrence)	S M E U Oth	37 1 7 6 5	mRNA analysis (Northern blot). Increased cyclin D1 transcript levels significantly associated with grade 1 and 2 tumours.	NS (PFI) / NS (DSS)
Worsley et al., (1999) (259)	43	3 LMP 40 EOC	S M E CC Mix U		Immunohistochemistry study. Increased cyclin D1 levels associated with borderline or well differentiated tumours.	Not investigated.

LMP: Low Malignant Potential (Borderline)
S: Serous
M: Mucinous
E: Endometrioid
CC: Clear Cell
U: Undifferentiated
Mix: Mixed Epithelial Cancer
Oth: Others (type not mentioned)
NS: Not significant
EOC: Epithelial Ovarian Cancer

Cyclin E gene amplification and protein overexpression correlates with advanced stage and poor grade in a small proportion of breast and colorectal cancers, suggesting its role as an adverse prognosticator in these cancers (195, 200). Very little is known about cyclin E expression in ovarian cancer. Gene amplification has been observed in approximately 20% of ovarian tumours and this correlates with increased cyclin E mRNA levels. Cyclin E mRNA or protein overexpression did not correlate with clinical outcome or other clinicopathological features of ovarian malignancy (271, 272). The only positive finding relating to cyclin E expression in ovarian cancer, is that expression appears to be high in clear cell carcinomas and low in serous subtypes of EOC.

2.3.4 pRb

Rb gene mutations have been described in a wide variety of human neoplasms including gall bladder, breast and lung cancers, and loss of pRb is associated with poor clinical outcome in these tumours (180-182). Allelic loss at the *Rb* locus occurs in 30-70% of ovarian cancers, although corresponding loss of pRb is rare (158, 183, 184, 273) possibly suggesting involvement of another gene at this locus. Most epithelial ovarian tumours, except mucinous cystadenocarcinomas (274, 275), show high pRb expression using immunohistochemical techniques and expression does not appear to co-segregate with other tumour characteristics (183). Only one study has demonstrated low expression of pRb to be associated with poor prognosis in ovarian cancer (184). In this study reduced pRb expression was an adverse prognostic marker of survival in FIGO stage 1 patients. Of the stage 1 patients with pRb expression >50%, all were alive at 5 years, in contrast to 50% survival of stage 1 patients with reduced pRb expression. Todd *et al.*, (2000), suggested that defects along the pRb/cyclinD1/p16^{Ink4a} pathway may play a role in the development of ovarian cancers (276). In a smaller immunohistochemical study the year previously Kusume *et al.*, (1999), showed abnormal expression in at least one of the four key genes in the pRb/cyclinD1/Cdk4/ p16^{Ink4a} pathway was adversely prognostic in patients with EOC (277).

2.4   Conclusion and Aims of Thesis

Many oncogenes and tumour suppressor genes have been implicated in the pathogenesis of ovarian cancer. Several of these genes are important in cell cycle regulation, in particular during G_1 to S phase transition of cellular division. The aberrant expression of a few of these cell cycle regulatory genes, e.g. p53, p21$^{Waf1/Cip1}$ and cyclin D1, have been studied in ovarian cancer, although these studies have reported conflicting results. In the majority, the number of tumours investigated is less than 100 and EOC are generally classified as a single entity. Moreover, the data relating to expression of other cell cycle genes, e.g. cyclin E, p27^{Kip1} and p16^{Ink4a}, in ovarian malignancy is limited.

The aim of this research was to extend the present literature by investigating the protein expression of seven cell cycle regulatory genes in a single subtype of invasive epithelial ovarian cancers, and determine if expression of these genes could be used as markers of clinical outcome. Serous cystadenocarcinomas is the most common type of epithelial ovarian tumour, and this histological subtype was chosen for our study. The genes selected for this study are critical in the two pathways, namely cyclin D1/CDK4/p16^{Ink4a} and cyclin E/CDK2/p27^{Kip1}, which converge on pRb. In addition, we also examined p21$^{Waf1/Cip1}$ that influences the progression of both pathways and p53 that transcriptionally regulates the expression of p21$^{Waf1/Cip1}$. A further aim of this study was to investigate the relationship of biomarkers to each other and to clinicopathological prognosticators of disease outcome.

Chapter Three   Methods and Materials

3.1   Patient Characteristics

Approval for this study was granted by the South Eastern Sydney Area Health Service Ethics Committee (Reference number: 00/115). The cohort was selected from a consecutive series of 272 patients treated for serous epithelial ovarian cancer at the Royal Hospital for Women, Sydney between 1988 and 1998. After excluding patients receiving primary treatment elsewhere (n=102), neoadjuvant therapy (n=22) and lack of representative tissue material (n=14), 141 patients with invasive serous ovarian cancer were identified for the present study. Census follow up was to March 2001, with a minimum follow up of 36 months.

Retrospective review of all patient files was performed to obtain pertinent data related to known clinical prognosticators, type of surgery undertaken, adjuvant therapy received, recurrence and survival. Clinical follow-up for this cohort of patients was obtained from the medical records and the NSW state register of Births, Deaths and Marriages. The gynaecology oncology practice at the Royal Hospital for Women is the largest in Sydney. Three gynaecology oncologists provide this service. Treatment of patients is standardised and reflects the most current method of practice. A definitive diagnosis is made on histopathological examination of resected tissue in accordance with WHO standards after primary laparotomy for suspected ovarian cancer. All tumours are staged according to the International Federation of Gynaecology and Obstetrics standards. Patients are followed post-operatively by their surgeons on a regular basis dependent upon disease progression.

The median age at diagnosis of patients entered in this study was 60.3 years (33-86 years). All patients underwent surgical resection as their primary diagnostic and treatment modality. One hundred and thirty two patients received combined adjuvant chemotherapy of cyclophosphamide or taxol and a platinum based agent, either cisplatinum or carboplatinum. Only 2 patients did not receive adjuvant

chemotherapy. One was found to have a well differentiated, stage Ia cancer and one with stage IIIc cancer who refused post-operative treatment. One hundred and twenty patients (89%) were diagnosed with FIGO stage III or greater disease. This rate is higher than the reported literature but is likely to represent the experience at a tertiary referral centre where more advanced and complicated cases are generally seen. The majority of tumours were moderately (48%) or poorly differentiated (47%). One hundred and two patients (76%) were debulked to residual tumour of less than 2 cm. Intra-operative ascites was present in 88 patients (66%). Confirmation of ascites could not be obtained in 1 case. Similarly the performance status of 12 patients was not recorded in the notes. Of the remaining patients, 86 had a GOG performance status of 0 (70%) as recorded in the notes. Seven patients died secondary to post-operative complications, and of other causes not related to their disease, and were excluded from disease-specific survival analysis. The 2-year and 5-year disease-specific survival rate in the remaining 134 patients was 67% and 31% respectively. One hundred and twelve patients (84%) suffered a relapse in whom the median time to recurrence was 16 months (6-132 months). Of these patients, 78% had a complete response of their disease with primary surgery and chemotherapy as defined by normalisation of CA125 levels or negative second look surgery. The remainder had either partial response (n=17) to treatment or no response (n=7) to treatment. The clinical and pathological characteristics of the patient cohort are listed in Table 3.1.

Table 3.1   Clinical and pathological characteristics of patients with disease-specific follow-up.

Characteristic	Patient number	Percentage
Age of patient		
≤65 years	84/134	62.7
>65 years	50/134	37.3
Stage of cancer		
1	10/134	7.5
2	4/134	3.0
3	102/134	76.1
4	18/134	13.4
Grade of tumour		
Well differentiated	7/134	5.2
Moderately differentiated	64/134	47.8
Poorly differentiated	63/134	47
Residual Disease		
≤2 cm	102/134	76.1
>2 cm	32/134	23.9
Performance Status		
0	86/122	70.5
1,2	36/122	29.5
Presence of Ascites		
No	45/133	33.8
Yes	88/133	66.2
Adjuvant Chemotherapy		
No	2/134	1.5
Yes	132/134	98.5
Disease progression		
No	22/134	16.4
Yes	112/134	83.6
Status		
Alive	46/134	34.3
Deceased	88/134	65.7

3.2   Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin embedded tissue samples sectioned at 4 microns and mounted on SuperFrost slides (Menzel-Glaser, Germany). The tissue samples were deparaffinised by soaking in xylene (two times for 5 minutes each) and rehydrated through graded ethanol. The Vectastain Elite Avidin-Biotin Complex kit (ABC kit, Vector Laboratories, California) was used as the detection system and colour development was obtained using 3,3-diaminobenzidine (DAB kit, Vector Laboratories, California). Counter-staining was undertaken with Whitlock's haemotoxylin, Scott's "blueing solution" with or without "light green" before dehydration through graded ethanol and xylene before cover slipping. Specific protocols for antigen retrieval, antibody dilution, and antigen staining are summarised below. Serial dilutions and manufacturer's instructions determined the antibody dilution resulting in the most appropriate staining pattern for ovarian tissue. The cells representing positive and negative controls were chosen from published literature with known expression of cell cycle regulatory molecules secondary to either amplification, mutations or loss of heterozygosity of the corresponding gene. Representative photomicrographs of positive and negative controls for each of the antigens are illustrated in Figure 3.1.

3.2.1   p53

Endogenous peroxidase activity was quenched using 3% hydrogen peroxide (H_2O_2) in methanol for 5 minutes. The sections were then boiled in a microwave oven in 0.01M citrate buffer (pH 6.0) for antigen retrieval. Twenty-percent normal horse serum (NHS) in 2% bovine serum albumin in tris-buffered saline (BSA/TBS) was subsequently used to block non-specific staining for 20 minutes. Slides were then incubated overnight at 4°C with an anti-p53 antibody. The sections were then sequentially incubated with biotinylated horse anti-mouse antibody (1:200 dilution BSA/TBS, Vector laboratories) for 30 minutes. Details of the primary antibody, and p53 positive and negative cell line controls are listed in Table 3.2.

Table 3.2 Anti-p53 antibody, dilution and positive and negative tissue controls.

p53		Comment	Reference
Clone	DO-7	Mouse monoclonal antibody	
Company	DAKO Corporation	Carpinteria, CA	
Dilution	1:500		
Controls			
Positive	DU145	Prostate cancer cell line	(278)
Negative	PC3	Prostate cancer cell line	

3.2.2 pRb

The tissue sections were boiled in a pressure cooker containing 1600 mls of 0.01M citrate buffer (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was quenched using 1.5% H_2O_2 in methanol for 10 minutes. Twenty-percent NHS in 2% TBS for 20 minutes was used to block non-specific staining. Slides were then incubated overnight at 4°C with an anti-pRb antibody. The sections were then sequentially incubated with biotinylated horse anti-mouse antibody (1:200 dilution TBS) for 30 minutes. Detection and colour development of the specimens was performed as described previously. Details of the primary antibody, and pRb positive and negative cell line controls are listed in Table 3.3.

Table 3.3 Anti-pRb antibody, dilution and positive and negative tissue controls

pRb		Comment	Reference
Clone	G3-245	Mouse monoclonal antibody	
Company	PharMingen International	San Diego, CA	
Dilution	1:200		
Controls			
Positive	MCF-7	Breast cancer cell line	(279)
Negative	MDA-MB-468	Breast cancer cell line	

3.2.3 p21$^{Waf1/Cip1}$

Endogenous peroxidase activity was quenched using 3% H_2O_2 in methanol for 10 minutes. The sections were then boiled in a microwave oven in 0.01M citrate buffer (pH 6.0) for antigen retrieval. Four-percent skimmed milk for 20 minutes followed by 40% NHS in 2% BSA/TBS for 20 minutes was used to block non-specific staining. Slides were then incubated overnight at 4°C with an anti-p21$^{Waf1/Cip1}$ antibody. The sections were then sequentially incubated with biotinylated horse anti-mouse antibody (1:200 dilution TBS) for 30 minutes. Details of the primary antibody, and p21$^{Waf1/Cip1}$ positive and negative cell line controls are listed in Table 3.4.

Table 3.4 Anti-p21$^{Waf1/Cip1}$ antibody, dilution and positive and negative tissue controls

p21$^{Waf1/Cip1}$		Comment	Reference
Clone	70	Mouse monoclonal antibody	
Company	Transduction laboratories	Lexington, KY	
Dilution	1:200		
Controls			
Positive	HMEC 184	Normal breast cell line	(279)
Negative	BT 549	Breast cancer cell line	

3.2.4 p27^{Kip1}, p16^{Ink4a}, cyclin E

The tissue sections were immersed in target retrieval solution, a modified citrate buffer (TRS, DAKO Corporation, Carpinteria) and the container placed in a boiling water bath for 30 minutes to perform antigen retrieval (TRS pH 6.1: p27^{Kip1} and cyclin E, TRS pH 9.9: p16^{Ink4a}). Endogenous peroxidase activity was quenched using 3% H_2O_2 in deionised water for 10 minutes. The sections were then incubated with primary antibody in concentrations as shown below for 30 minutes. Biotinylated secondary antibody (DAKO Corporation, Carpinteria) was subsequently applied followed by enzyme conjugated streptavidin and substrate chromogen (DAKO Corporation, Carpinteria) in accordance with the manufacturer's instructions.

Details of the primary antibody and positive and negative tissue controls for each of the antigens are shown in Tables 3.5 – 3.7.

Table 3.5 Anti-p27^{Kip1} antibody, dilution and positive and negative tissue controls

p27^{Kip1}		Comment	Reference
Clone	57	Mouse monoclonal antibody	
Company	Transduction laboratories	Lexington, KY	
Dilution	1:200		
Controls			
Positive	BT 474	Breast cancer cell line	(279)
Negative	HMEC 184	Normal breast cell line	

Table 3.6 Anti-p16^{Ink4a} antibody, dilution and positive and negative tissue controls

p16^{Ink4a}		Comment	Reference
Clone	16 PO4	Mouse monoclonal antibody	
Company	NeoMarkers	Fremont, CA	
Dilution	1:200		
Controls			
Positive	MDA-MB-157	Breast cancer cell line	(279, 280)
Negative	MDA-MB-231	Breast cancer cell line	(280)

Table 3.7 Anti-cyclin E antibody, dilution and positive and negative tissue controls

Cyclin E		Comment	Reference
Clone	HE 12	Mouse monoclonal antibody	
Company	PharMingen International	San Diego, CA	
Dilution	1:50		
Controls			
Positive	MDA-MB157	Breast cancer cell line	(281)
Negative	Salivary Gland	Normal tissue	

3.2.5 Cyclin D1

The tissue sections were boiled in a pressure cooker containing 1600 mls of EDTA (pH 8.0) to enhance antigen retrieval. Endogenous peroxidase activity was quenched using 3% H_2O_2 in deionised water for 10 minutes. A biotin blocking system (DAKO Corporation, Denmark) was used to block non-specific staining. The sections were then incubated with primary antibody in concentrations as shown below for 30 minutes. Biotinylated secondary antibody (DAKO Corporation, Carpinteria) was subsequently applied followed by enzyme conjugated streptavidin and substrate chromogen (DAKO Corporation, Carpinteria) in accordance with the manufacturer's instructions. Details of the primary antibody, and cyclin D1 positive and negative cell line controls are listed in Table 3.8.

Table 3.8 Anti-cyclin D1 antibody, dilution and positive and negative tissue controls

Cyclin D1		Comment	Reference
Clone	DCS-6	Mouse monoclonal antibody	
Company	DAKO Corporation	Carpinteria, CA	
Dilution	1:300		
Controls			
Positive	MCF-7M	Breast cancer cell line	(195)
Negative	HBL 100	Normal breast cell line	

(A) p53-

(B) p53+

(C) p21$^{\text{Waf1/Cip1}}$-

(D) p21$^{\text{Waf1/Cip1}}$+

(E) p27$^{\text{Kip1}}$-

(F) p27$^{\text{Kip1}}$+

(G) cyclin D1-
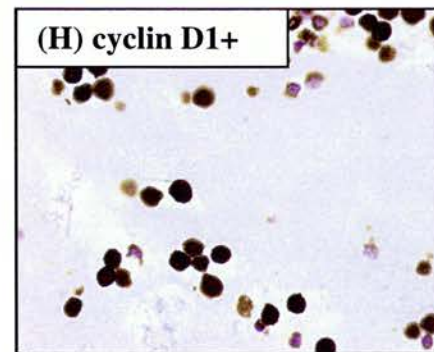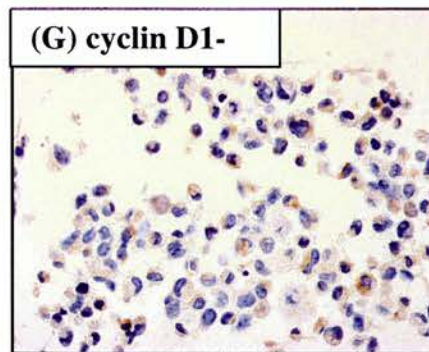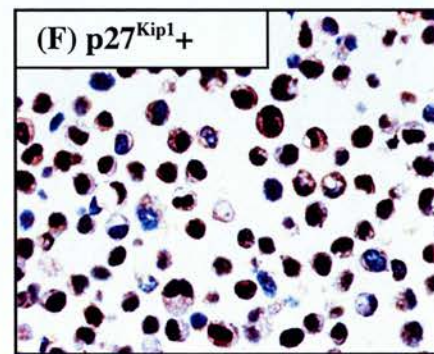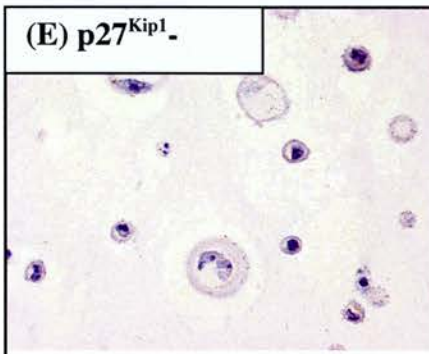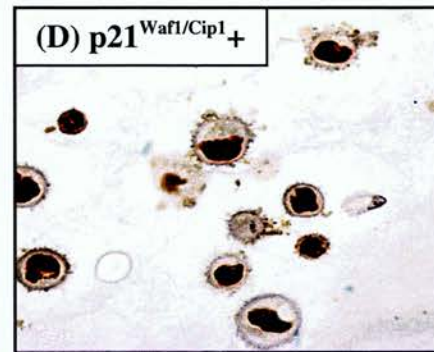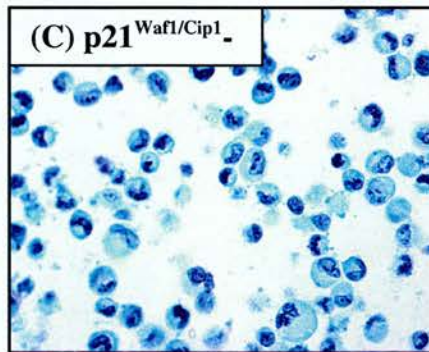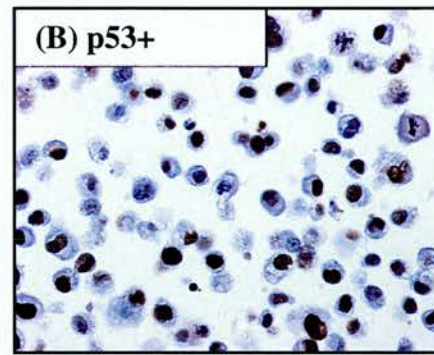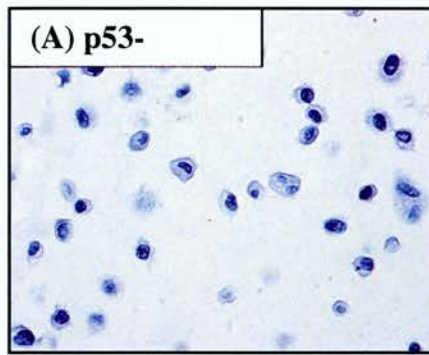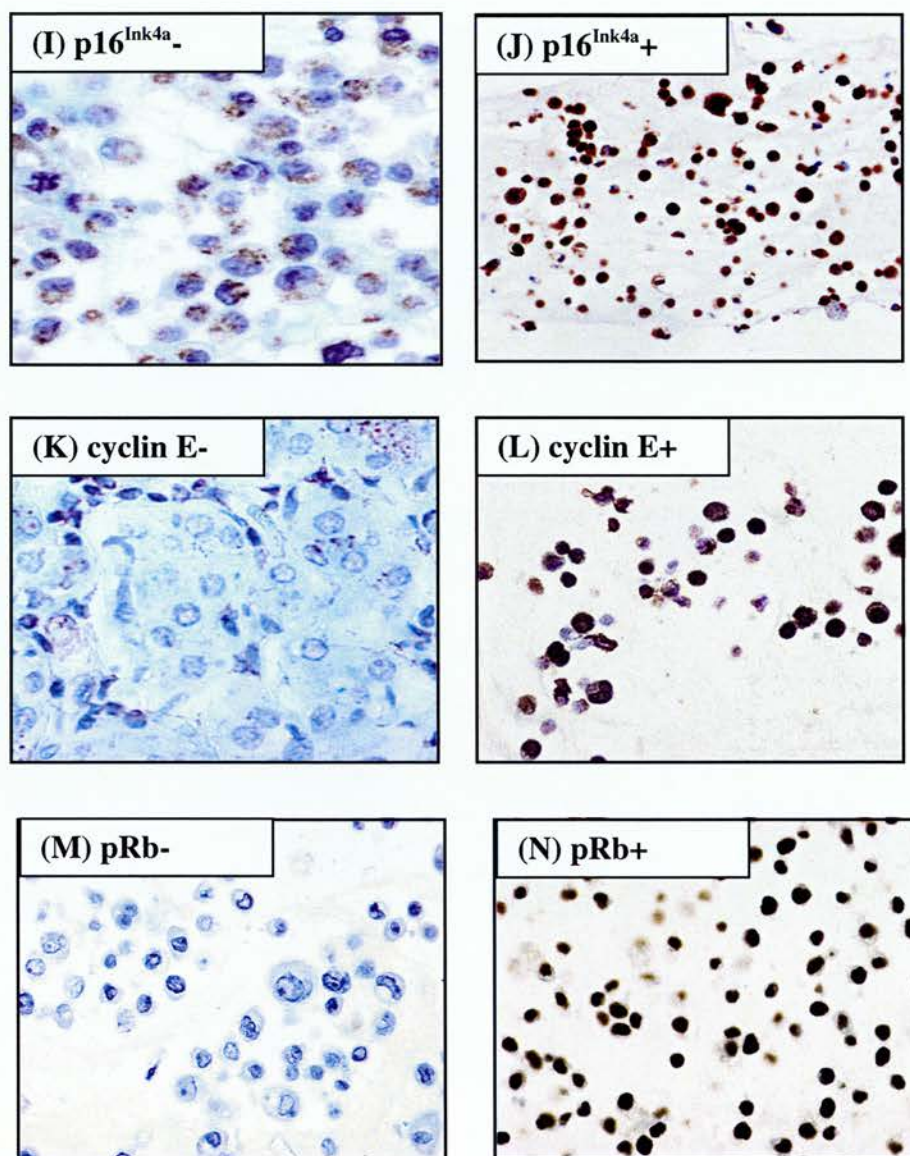
(H) cyclin D1+

Figure 3.1 (**A**) p53 – cell line control, PC 3, (**B**) p53 + cell line control, DU 145, (**C**) p21$^{Waf1/Cip1}$ – cell line control, BT 549, (**D**) p21$^{Waf1/Cip1}$ + cell line control, HMEC 184, (**E**) p27^{Kip1} – cell line control, HMEC 184, (**F**) p27^{Kip1} + cell line control, BT 474, (**G**)cyclin D1– cell line control, HBL 184, (**H**) cyclin D1 + cell line control, MCF-7, (**I**) p16^{Ink4a} – cell line control, MDA-MB-231, (**J**) p16^{Ink4a} + cell line control, MDA-MB-157, (**K**) cyclin E – tissue control, salivary gland, (**L**) cyclin E + cell line control, MDA-MB-157, (**M**) pRb – cell line control, MDA-MB-468, (**N**) pRb + cell line control, MCF-7.

Abbreviations
- Negative
+ Positive

3.3   Analysis

3.3.1 Scoring

Two separate observers, including a qualified pathologist, assessed the degree and pattern of tissue staining of molecular markers for each case. Standardisation of scoring was achieved by comparison of scores between observers and any discrepancies were resolved by consensus. Scores were given as an overall percentage of positive nuclear staining within 10 representative areas of the tumour sample at x400 magnification. The pattern of immunostaining for all the antigens was homogenous in this tissue cohort. The percentage values above which immunostaining was considered as positive for each of the antigens was based on reports from the published literature. There is a wide range of cut-off values above which immunostaining is considered as positive staining in the literature, and we have tried where possible, to standardise our scoring based on the majority consensus view of what is considered as positive expression. Table 3.9 summarises the percentage of nuclear staining above which positive staining was considered for each of the antigens studied.

Table 3.9   Cut-off values for positive antigen staining with associated references

	p53	p21$^{Waf1/Cip1}$	p27^{Kip1}	p16^{Ink4a}	cyclin E	cyclin D1	pRb
Positive Staining (% tumour nuclei stained)	>10%	>10%	>65%	>15%	>10%	>10%	>20%
Reference	(233, 238)	(196, 233)	(257, 259)	(263)	(266)	(266)	(277)

3.3.2 Statistics

An electronic relational database was constructed using Filemaker Pro 4.0 (Claris Corporation, North Ryde, Australia). This database was designed during the course of this study, intermittently modified and will be used as an essential resource for future investigations on this cohort. The information entered was appropriately coded, de-identified and password protected as per ethics guidelines (approved by the South Eastern Sydney Area Health Service Ethics Committee). The important clinicopathological features that were recorded in the database included; date of diagnosis, age at diagnosis, date of last follow-up or date of death as appropriate, date of disease progression, pre-operative and follow-up CA125 levels, pre-operative performance status, details of the primary laparotomy, presence of intra-operative ascites, volume of residual disease, FIGO stage, type and extent of post-operative chemotherapy, details of follow-up investigations that including radiology and second-look surgery, and pathological characteristics of the tumour including type and differentiation. The percentage of cancer cell nuclei stained for the antigens examined were also recorded in this database. For statistical analysis, the information was exported onto appropriate spreadsheets for integration with Statview 4.5 software (Abacus concepts, Berkley, California).

Clinicopathological features reported in the published literature (section 1.4) as being important determinants of clinical outcome in ovarian cancer include; volume of post-operative residual disease, presence of ascites performance status, FIGO stage, tumour grade, and to a lesser extent, age of the patient at the time of diagnosis and pre-operative serum CA125 level, and CA125 levels prior to the 4th cycle of chemotherapy. Disease-specific survival was calculated from the date of primary laparotomy to date of death or date of last follow up. The progression-free interval was calculated from the date of primary laparotomy to the date of recurrence as specified by biochemical, radiological or surgical relapse. Patients were categorised as having a complete response to primary surgery and adjuvant chemotherapy, as defined by normalisation of CA125 levels or negative second look surgery. Univariate survival analysis according to these parameters and expression of

molecular markers in this cohort of serous epithelial ovarian tumours, was determined using the Kaplan-Meier method and Cox proportional hazards model. Predictive variables of disease outcome on univariate analysis were incorporated into multivariate analysis using Cox proportional hazards modelling, to identify factors with independent significance in determining the clinical outcome of patients.

In this study the relationships between the molecular markers themselves and the correlation between the molecular markers and various clinical and pathological parameters was also determined. These analyses were performed using chi-square, Fisher's exact test and simple regression where appropriate. A p-value of <0.05 was accepted as statistically significant.

Chapter Four   Results

4.1   Summary

The expression of p53, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, cyclin D1, cyclin E, $p16^{Ink4a}$ and pRb were evaluated by immunohistochemistry on 134 primary serous ovarian cancers. Clinicopathological determinants of poor clinical outcome (reduced disease-specific and progression-free survival) included RD greater 2 cm, presence of ascites, poor performance status (1, 2), advanced FIGO stage (III, IV) and increasing tumour grade (moderately and poorly differentiated).

Overexpression of p53 occurred in 59% of tumours and was significantly associated with increasing tumour grade (p=0.03), loss of $p21^{Waf1/Cip1}$ expression (p=0.006), reduced DSS (p=0.02) and shorter PFI (p=0.004). Similarly, loss of $p21^{Waf1/Cip1}$ was demonstrated in 77% of cancer specimens and was also associated with increasing tumour grade (p=0.002) and shorter disease-specific (p=0.02) and progression-free survival (p=0.01). Although an association was observed between the expression of p53 and $p21^{Waf1/Cip1}$, when various combinations of p53 and $p21^{Waf1/Cip1}$ expression were analysed, patients whose tumours displayed overexpression of p53 with concurrent loss of $p21^{Waf1/Cip1}$ had a significantly shorter DSS (p=0.0008) and PFI (p=0.0001) than patients with other combinations of these two molecules. Overexpression of cyclin D1 occurred in 19% of tumours and was associated with reduced DSS (p=0.03). Reduced expression of $p27^{Kip1}$ was observed in 67% of tumours and was associated with increasing tumour grade (p=0.04), expression of $p21^{Waf1/Cip1}$ (p=0.006) and cyclin D1 (p=0.03) and shorter DSS (p=0.05) and PFI (0.05). On multivariate analysis using the Cox Proportional Hazards Model, overexpression of cyclin D1 and a combination of p53 overexpression with concurrent loss of $p21^{Waf1/Cip1}$ were both independently predictive of reduced DSS, when adjusted for volume of RD, PS and presence of ascites. p53 overexpression with loss of $p21^{Waf1/Cip1}$ also retained independent significance as a prognostic factor for shorter PFI when considered with the same clinical characteristics already mentioned.

On further analysis of p53, a differential staining pattern was observed across the tumour cohort. Less that 5% of p53 nuclear staining was observed in 40% (n=53) of tumours, greater than 50% staining in 41% (n=55) of specimens with the remainder (19% (n=26)) demonstrating intermediate expression, i.e. between 5% and 50%. On Kaplan-Meier survival analysis, only 8% of patients (n=2) that had intermediate expression of p53 in their tumour specimens were alive at 5 years, in contrast to 5-year survival rates of 36% and 43% in those with high expression (>50%) and low expression (<5%) of p53 respectively. This difference was highly significant (p < 0.0001).

4.2   Characteristics of the study cohort and clinicopathological determinants of disease outcome

The clinical and pathological characteristics of the study cohort have been previously described in section 3.1. Consistent with published data, the presence of ascites, large volume of residual disease (>2 cm), advanced stage (III, IV), increasing tumour grade (2,3) and worse performance status (1,2) were all significant determinants of reduced disease-specific survival (DSS) and 5-year progression-free interval (PFI). However, in the analyses of stage and grade of tumour the confidence intervals were large due to a small number of cases with well differentiated tumours (n=7), and patients with early stage disease (I & II, n=14), and consequently these results need to be interpreted with caution. Moreover, there was no survival advantage for patients with moderately differentiated tumours (n=64) as compared to those with poorly differentiated ones (n=63). Similarly, there was no difference in DSS and PFI of patients when analysis was stratified for stage III vs stage IV tumours. Again this may be due to the small number of patients with stage IV disease (n=18). In addition, patients could not be stratified by substage of disease predominantly due to the size of the cohort. The results of univariate analysis using the Cox Proportional Hazards Model, of clinicopathological characteristics of patients to disease-specific and progression-free survival are summarised in Table 4.1.

Table 4.1   Univariate analysis of relationships between clinicopathological characteristics and disease-specific survival and progression-free interval.

Clinical Parameter	Relative risk	(DSS)✥ 95% CI	p-value	Relative risk	(5-year PFI)✱ 95% CI	p-value
Residual disease ≤2cm vs > 2cm	3.05	1.92 – 4.86	**<0.0001**	2.98	1.82 – 4.40	**<0.0001**
Stage I-II vs III-IV	4.35	1.59 –11.90	**0.004**	3.33	1.61 – 7.52	**0.001**
Grade 1 vs 2-3	5.54	1.32 –23.26	**0.02**	2.34	1.06 –10.58	**0.05**
Ascites Yes vs No	2.28	1.39 – 3.74	**0.0006**	2.04	1.34 – 3.19	**0.0009**
Performance status: 0 vs 1-2	2.15	1.35 – 3.44	**0.001**	1.58	1.03 – 2.43	**0.03**
Age ≤65 vs > 65	1.31	0.67 – 1.61	0.87	0.94	0.65 – 1.43	0.84
CA125 ≤500 vs > 500	0.77	0.45 – 1.34	0.36	0.88	0.54 – 1.43	0.60

✥ Disease-specific survival was calculated from date of primary laparotomy to the date of disease-specific death or date of last follow up.

✱ 5-year progression-free interval was calculated from the date of primary laparotomy to date of disease progression. Data census was at 5 years from date of diagnosis.

4.3   Cell cycle gene expression in ovarian cancer and correlation between patterns of expression and clinicopathological characteristics of the study cohort

The expression of p53, $p21^{Waf1/Cip1}$, $p27^{Kip1}$, cyclin D1, cyclin E, $p16^{Ink4a}$ and pRb were evaluated by immunohistochemistry on 134 primary serous ovarian cancers. Expression was quantified on the basis of the percentage of positively stained nuclei and allocated a score to represent the degree of overexpression.   The median percentage of immunostaining, percentage values above which expression was deemed positive and number of specimens displaying positive staining is shown in Table 4.2.

Table 4.2 Immunohistochemical staining of cell cycle genes studied in serous epithelial ovarian cancer

	p53	p21[Waf1/Cip1]	p27[Kip1]	p16[Ink4a]	cyc E	cyc D1	pRb
Range of staining (% of positive nuclei)	0-100	0-60	0-95	0-100	0-95	0-60	0-100
Median staining (% of positive nuclei)	27	4	40	15	20	2	70
Cut-off Value (positive staining)	>10	>10	>65	>15	>10	>10	>20
Positive tumours (percentage)	79 (59)	31 (23)	45 (33)	74 (55)	92 (68)	25 (19)	(79)

4.3.1 Clinicopathological and molecular correlates of p53 protein expression

Increased expression of p53 protein in tumour specimens was considered if >10% of cancer cell nuclei stained positive for p53 protein. The pattern of nuclear staining was generally intense and homogenous in the invasive tumour. Representative photomicrographs of tumour tissue showing positive and negative staining for p53 are presented in Figure 4.1. Expression of p53 correlated to grade (p=0.03), with 14% of well-differentiated tumours staining positive for p53, increasing to approximately 50% in the moderately and poorly differentiated group. There was no correlation with between p53 expression and stage (p=0.70), volume of residual disease (p=0.81) or presence of ascites (p=0.17).

There was no correlation between the expression of p27[Kip1], cyclin D1, and pRb and nuclear accumulation of p53. However, there was a significant association between expression of p53 and p21[Waf1/Cip1]. Of the tumours that overexpressed p53, 14% had a concurrent increase in p21[Waf1/Cip1] protein levels, in contrast to p53 negative tumours where 40% were p21[Waf1/Cip1] positive (p=0.006). Similarly there was a significant negative association between p53 expression and p16[Ink4a] expression. Of the p53 positive tumours, 37% demonstrated positive p16[Ink4a] expression, whereas in p53 negative tumours there was positive p16[Ink4a] expression in 62% of tumours (p=0.004). There was also a weak positive correlation between overexpression of

p53 and positive cyclin E expression. Sixty seven percent of p53 positive tumours were also positive for cyclin E expression, in contrast to p53 negative tumours where 50% of tumours were cyclin E positive (p=0.04). These associations are demonstrated in Table 4.3.

Table 4.3   Correlation between p53 expression and clinicopathological and molecular parameters

			p53 (n=134)		
		Number	Positive > 10%	Negative ≤ 10%	p-value
Stage	I	10 (8%)	4 (40%)	6	
(n=134)	II	4 (3%)	2 (50%)	2	0.70
	III	102 (76%)	60 (59%)	42	
	IV	18 (13%)	10 (55%)	8	
Grade	1	7 (5%)	1 (14%)	6	
(n=134)	2	64 (48%)	41 (64%)	23	**0.03**
	3	63 (47%)	34 (54%)	29	
Ascites	No	45 (34%)	21 (49%)	22	0.17
(n=133)	Yes	88 (66%)	53 (61%)	34	
RD	≤ 2 cm	103 (77%)	59 (57%)	44	0.81
(n=134)	> 2 cm	31 (23%)	17 (54%)	14	

	p53 (n=134)		
	Positive > 10%	Negative ≤ 10%	p-value
p21$^{Waf1/Clp1}$ (n=134)			
Positive > 10%	11	20	**0.006**
Negative ≤ 10%	65	38	
p27^{Klp1} (n=134)			
Positive > 65%	24	22	0.44
Negative ≤ 65%	52	36	
p16^{Ink4a} (n=134)			
Positive > 15%	28	36	**0.004**
Negative ≤ 15%	48	22	
Cyclin D1 (n=134)			
Positive > 10%	13	12	0.60
Negative ≤ 10%	63	46	
Cyclin E (n=134)			
Positive > 10%	51	29	**0.04**
Negative ≤ 10%	25	29	
pRb (n=134)			
Positive > 20%	56	44	0.77
Negative ≤ 20%	20	14	
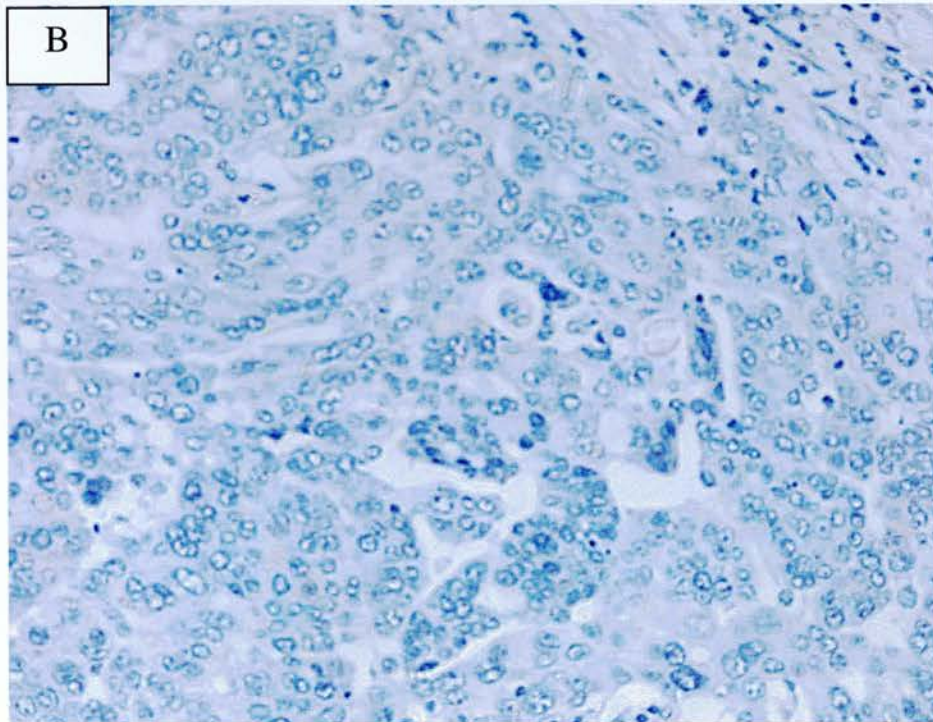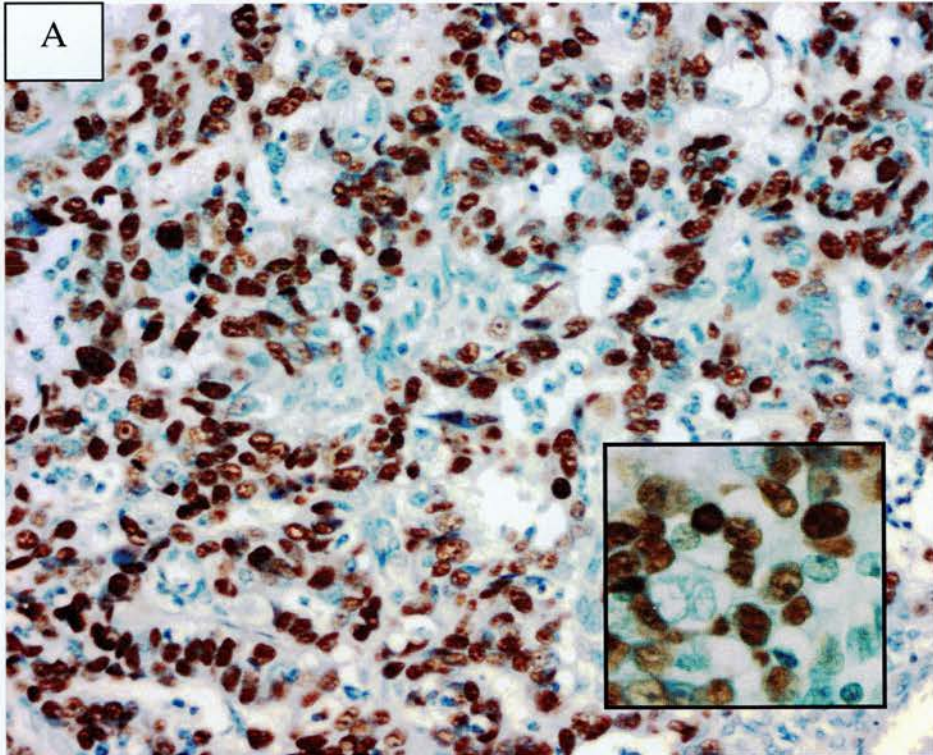
Figure 4.1 (A) Marked overexpression of p53 in a poorly differentiated serous ovarian cancer. Insert at x400 magnification. (B) p53 negative tumour nuclei in a poorly differentiated serous ovarian cancer. (x200 magnification)

4.3.2 Clinicopathological and molecular correlates of p21^{Waf1/Cip1} protein expression

Overexpression of $p21^{Waf1/Cip1}$ in tumour specimens was considered if >10% of cancer cell nuclei stained positive for $p21^{Waf1/Cip1}$ protein. The pattern of nuclear staining was uniform and intense in the invasive tumour. Positive and negative immunohistochemical staining of $p21^{Waf1/Cip1}$ in tumour tissue is demonstrated in Figure 4.2. Expression of $p21^{Waf1/Cip1}$ correlated strongly to grade (p=0.002), with 71% of well differentiated tumours staining positive for $p21^{Waf1}$, decreasing dramatically to 27% and 14% in the moderately and poorly differentiated groups respectively. There was no correlation between the expression of $p21^{Waf1/Cip1}$ and volume of residual disease (p=0.69), presence of ascites (p=0.28) or stage, although there was a trend towards decreased expression with increasing stage but this did not reach statistical significance (p=0.07)

There was no correlation between the expression of $p16^{Ink4a}$, cyclin D1, cyclin E, and pRb and nuclear accumulation of $p21^{Waf1/Cip1}$. However, there was a significant correlate between expression of p53 and $p21^{Waf1/Cip1}$ as mentioned in section 4.1. Similarly there was a significant positive association between $p21^{Waf1/Cip1}$ expression and $p27^{Kip1}$ expression. Of the $p21^{Waf1/Cip1}$ positive tumours, 55% demonstrated positive $p27^{Kip1}$ expression, whereas in $p21^{Waf1/Cip1}$ negative tumours there was positive $p27^{Kip1}$ expression in only 28% of tumours (p=0.006). These associations are shown in Table 4.4.

Table 4.4 Correlation between p21$^{Waf1/Cip1}$ expression and clinicopathological and molecular parameters

p21$^{Waf1/Cip1}$ (n=134)					
		Number	Positive >10%	Negative ≤10%	p-value
Stage	I	10 (8%)	3 (30%)	7	
(n=134)	II	4 (3%)	3 (75%)	1	0.07
	III	102 (76%)	22 (21%)	80	
	IV	18 (13%)	3 (17%)	15	
Grade	1	7 (5%)	5 (71%)	2	
(n=134)	2	64 (48%)	17 (27%)	47	**0.002**
	3	63 (47%)	9 (14%)	54	
Ascites	No	45 (34%)	13 (29%)	32	0.28
(n=133)	Yes	88 (66%)	18 (20%)	70	
RD	≤ 2 cm	103 (77%)	23 (22%)	80	0.69
(n=134)	> 2 cm	31 (23%)	8 (26%)	23	

p21$^{Waf1/Cip1}$ (n=134)			
	Positive >10%	Negative ≤10%	p-value
p53 (n=134)			
Positive > 10%	11	65	**0.006**
Negative ≤ 10%	20	38	
p27^{Kip1} (n=134)			
Positive > 65%	17	29	**0.006**
Negative ≤ 65%	14	74	
p16^{Ink4a} (n=134)			
Positive > 15%	16	48	0.71
Negative ≤ 15%	15	55	
Cyclin D1 (n=134)			
Positive > 10%	4	21	0.43
Negative ≤ 10%	27	82	
Cyclin E (n=134)			
Positive > 10%	16	64	0.29
Negative ≤ 10%	15	39	
pRb (n=134)			
Positive > 20%	21	79	0.31
Negative ≤ 20%	10	24	
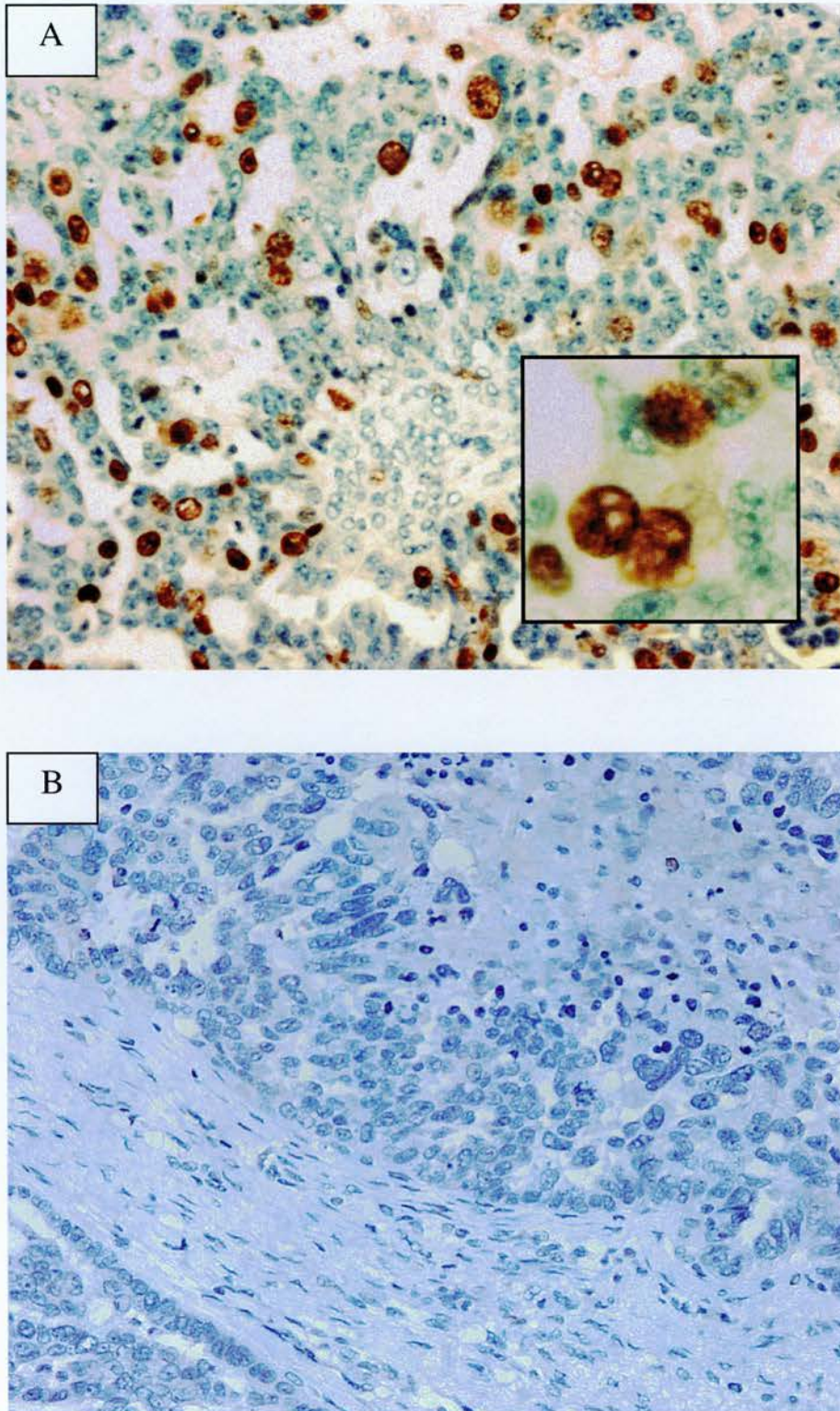
Figure 4.2 (A) Moderate expression of p21$^{\text{Waf1/Cip1}}$ in a well differentiated serous tumour. Insert represents area at x400 magnification. (B) Poorly differentiated tumour negative for p21 $^{\text{Waf1/Cip1}}$ expression. (x200 magnification)

4.3.3 Clinicopathological and molecular correlates of p27^{Kip1} protein expression

High expression of p27^{Kip1} in tumour specimens was considered if >65% of cancer cell nuclei stained positive for p27^{Kip1} protein. The pattern of nuclear staining was uniform and intense in the invasive tumour. Positive and negative immunohistochemical staining of p27^{Kip1} in tumour tissue is demonstrated in Figure 4.3. Expression of p27$^{Cip1/Kip1}$ correlated weakly to grade (p=0.04), with 57% of well differentiated tumours staining positive for p27^{Kip1}, decreasing to 42% and 23% in the moderately and poorly differentiated groups respectively. There was no correlation between the expression of p27^{Kip1} and volume of residual disease (p=0.48), presence of ascites (p=0.39) or tumour stage (p=0.32)

No correlation was observed between the expression of p53, p16^{Ink4a}, cyclin E, and pRb and nuclear accumulation of p27^{Kip1}. There was a significant correlation between expression levels of p27^{Kip1} and p21$^{Waf1/Cip1}$ as mentioned in section 4.2. There was a weakly significant negative association between p27^{Kip1} and cyclin D1 expression. Of the p27^{Kip1} positive tumours, 9% demonstrated positive cyclin D1 expression, whereas in p27^{Kip1} negative tumours there was positive cyclin D1 expression in 24% of tumours (p=0.03). These associations are demonstrated in Table 4.5.

Table 4.5 Correlation between p27^{Kip1} expression and clinicopathological and molecular parameters

p27^{Kip1} (n=134)		Number	Positive > 65%	Negative ≤ 65%	p-value
Stage	I	10 (8%)	4 (40%)	6	
(n=134)	II	4 (3%)	3 (75%)	1	0.325
	III	102 (76%)	34 (33%)	34	
	IV	18 (13%)	5 (28%)	5	
Grade	1	7 (5%)	4 (57%)	3	
(n=134)	2	64 (48%)	27 (42%)	37	**0.04**
	3	63 (47%)	15 (23%)	48	
Ascites	No	45 (34%)	12 (29%)	32	0.39
(n=133)	Yes	88 (66%)	32 (36%)	56	
RD	≤ 2 cm	103 (77%)	37 (36%)	66	0.48
(n=134)	> 2 cm	31 (23%)	9 (29%)	22	

p27^{Kip1} (n=134)	Positive > 65%	Negative ≤ 65%	p-value
p53 (n=134)			
Positive > 10%	24	52	0.44
Negative ≤ 10%	22	36	
p21$^{Waf1/Cip1}$ (n=134)			
Positive > 10%	17	14	**0.006**
Negative ≤ 10%	29	74	
p16^{Ink4a} (n=134)			
Positive > 15%	23	41	0.71
Negative ≤ 15%	23	47	
Cyclin D1 (n=134)			
Positive > 10%	4	21	**0.03**
Negative ≤ 10%	42	67	
Cyclin E (n=134)			
Positive > 10%	30	50	0.34
Negative ≤ 10%	16	38	
pRb (n=134)			
Positive > 20%	32	68	0.33
Negative ≤ 20%	14	20	
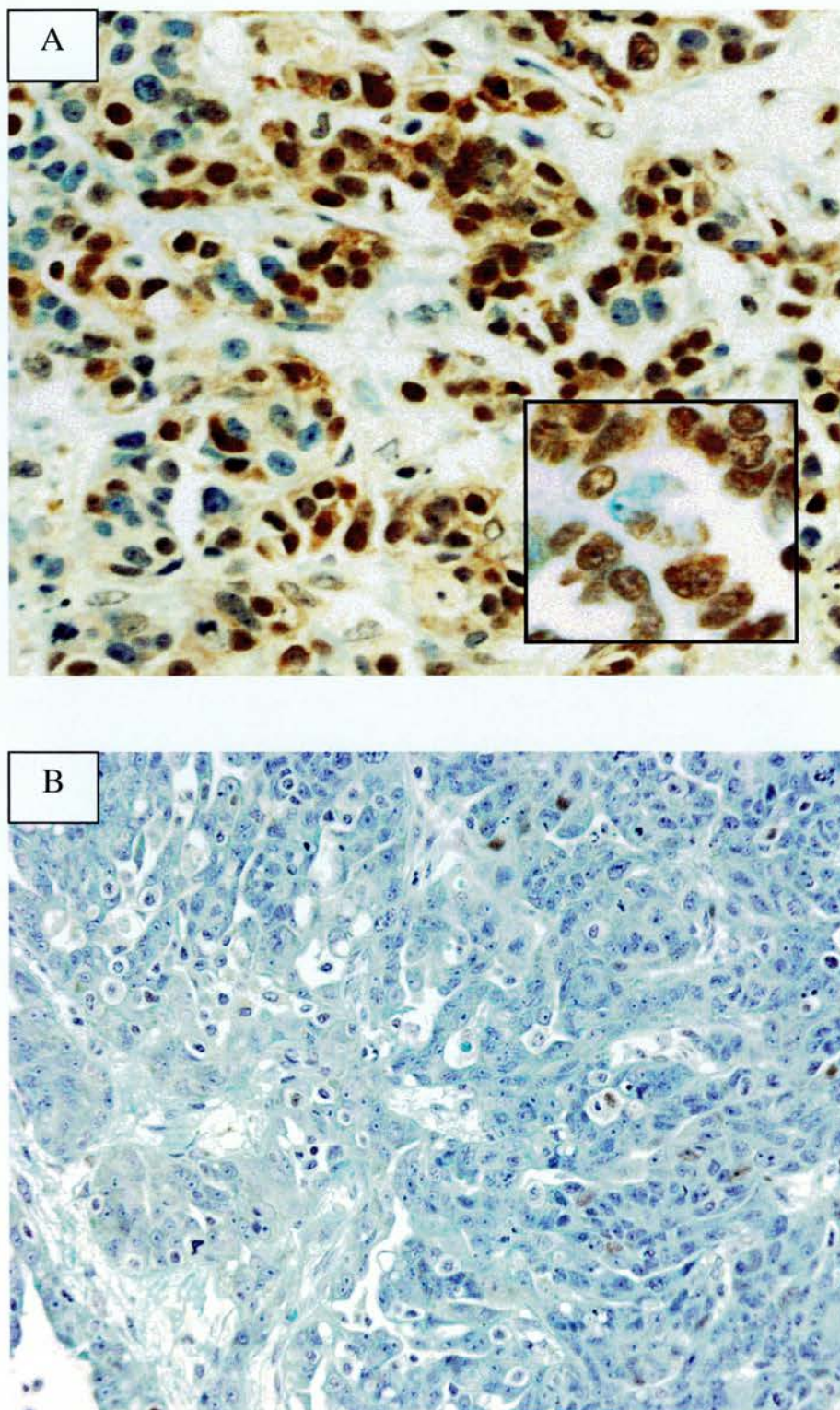
Figure 4.3 (A) High p27^{Kip1} expression in a poorly differentiated serous ovarian cancer. Insert represents area at x400 magnification. (B) Absent p27^{Kip1} expression in a poorly differentiated serous ovarian cancer. (x200 magnification).

4.3.4 Clinicopathological and molecular correlates of cyclin D1 protein expression

High expression of cyclin D1 in tumour specimens was considered if >10% of cancer cell nuclei stained positive for cyclin D1 protein. The pattern of nuclear staining was uniform in the invasive tumour. Representative photomicrographs of tumour tissue showing positive and negative staining for cyclin D1 are presented in Figure 4.4. There was no correlation between the expression of cyclin D1 and volume of residual disease (p=0.68), presence of ascites (p=0.49), stage (p=0.86), or tumour grade (p=0.21)

There was no correlation between the expression of p53, $p21^{Waf1/Cip1}$, $p16^{Ink4a}$, cyclin E, and pRb and nuclear accumulation of cyclin D1. There was a significant correlation between expression levels of cyclin D1 and $p27^{Kip1}$ as mentioned in section 4.3. The results are shown in Table 4.6.

Table 4.6   Correlation between cyclin D1expression and clinicopathological and molecular parameters.

		Cyclin D1 (n=134)			
		Number	Positive > 10%	Negative ≤ 10%	p-value
Stage	I	10 (8%)	1 (10%)	9	
(n=134)	II	4 (3%)	1 (25%)	3	0.86
	III	102 (76%)	19 (19%)	83	
	IV	18 (13%)	4 (22%)	14	
Grade	1	7 (5%)	0 (0%)	7	
(n=134)	2	64 (48%)	10 (16%)	54	0.21
	3	63 (47%)	15 (23%)	48	
Ascites	No	45 (34%)	7 (16%)	38	0.49
(n=133)	Yes	88 (66%)	18 (20%)	70	
RD	≤ 2 cm	103 (77%)	20 (19%)	83	0.68
(n=134)	> 2 cm	31 (23%)	5 (16%)	26	

Table 4.6   Continued.

Cyclin D1 (n=134)			
	Positive > 10%	Negative ≤ 10%	p-value
p53 (n=134)			
Positive > 10%	13	63	0.60
Negative ≤ 10%	12	46	
p21$^{Waf1/Cip1}$ (n=134)			
Positive > 10%	4	27	0.43
Negative ≤ 10%	21	82	
p27^{Kip1} (n=134)			
Positive > 65%	4	42	**0.03**
Negative ≤ 65%	21	67	
p16^{Ink4a} (n=134)			
Positive > 15%	9	55	0.19
Negative ≤ 15%	16	54	
Cyclin E (n=134)			
Positive > 10%	12	68	0.18
Negative ≤ 10%	13	41	
pRb (n=134)			
Positive > 20%	20	80	0.49
Negative ≤ 20%	5	29	
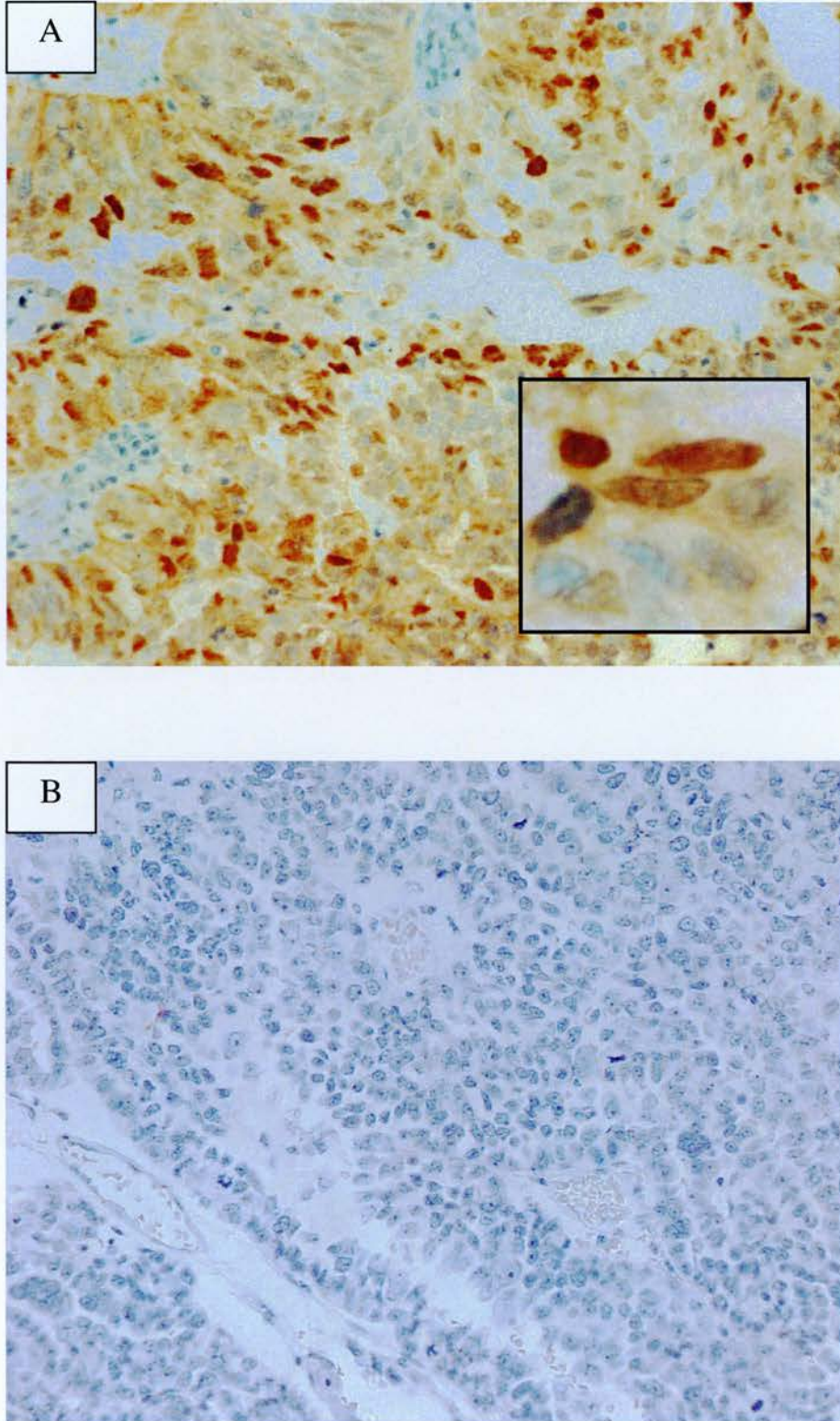
Figure 4.4 (A) cyclin D1 positive tumour nuclei in a poorly differentiated serous ovarian cancer. Insert represents nuclear staining at x400 magnification. (B) cyclin D1 negative tumour nuclei in a poorly differentiated serous ovarian cancer. (x200 magnification).

4.3.5 Clinicopathological and molecular correlates of cyclin E protein expression

High expression of cyclin E in tumour specimens was considered if >10% of cancer nuclei stained positive for cyclin E protein. The pattern of nuclear staining was uniform and intense in the invasive tumour. Representative photomicrographs of tumour tissue showing positive and negative staining for cyclin E are presented in Figure 4.5. Expression of cyclin E correlated weakly to grade (p=0.03), with 14% of well differentiated tumours staining positive for cyclin E, increasing to 59% and 65% in the moderately and poorly differentiated group respectively. There was no correlation with volume of residual disease (p=0.53), presence of ascites (p=0.73) or with stage (p=0.45)

There was no correlation between the expression of $p21^{Waf1/Cip1}$, $p27^{Kip1}$, $p16^{Ink4a}$, cyclin D1, and pRb and nuclear accumulation of cyclin E. There was a significant positive correlation between expression levels of cyclin E and p53 as mentioned in section 4.3.1. These associations are demonstrated in Table 4.7.

Table 4.7   Correlation between cyclin E expression and clinicopathological and molecular parameters

		cyclin E (n=134)			
		Number	Positive > 10%	Negative ≤10%	p-value
Stage	I	10 (8%)	7 (70%)	3	
(n=134)	II	4 (3%)	1 (25%)	3	0.45
	III	102 (76%)	62 (60%)	40	
	IV	18 (13%)	10 (55%)	8	
Grade	1	7 (5%)	1 (14%)	6	
(n=134)	2	64 (48%)	38 (59%)	26	**0.03**
	3	63 (47%)	41 (65%)	22	
Ascites	No	45 (34%)	28 (62%)	17	0.73
(n=133)	Yes	88 (66%)	52 (59%)	36	
RD	≤ 2 cm	103 (77%)	60 (58%)	43	0.53
(n=134)	> 2 cm	31 (23%)	20 (64%)	11	

Table 4.7 Continued.

cyclin E (n=134)			
	Positive > 10%	**Negative ≤ 10%**	**p-value**
p53 (n=134)			
Positive > 10%	51	25	**0.045**
Negative ≤ 10%	29	29	
p21[Waf1/Cip1] (n=134)			
Positive > 10%	16	15	0.29
Negative ≤ 10%	64	39	
p27[Kip1] (n=134)			
Positive > 65%	30	16	0.34
Negative ≤ 65%	50	38	
p16[Ink4a] (n=134)			
Positive > 15%	41	23	0.33
Negative ≤ 15%	39	31	
cyclinD1 (n=134)			
Positive > 10%	12	13	0.18
Negative ≤ 10%	68	41	
pRb (n=134)			
Positive > 20%	58	42	0.49
Negative ≤ 20%	22	12	
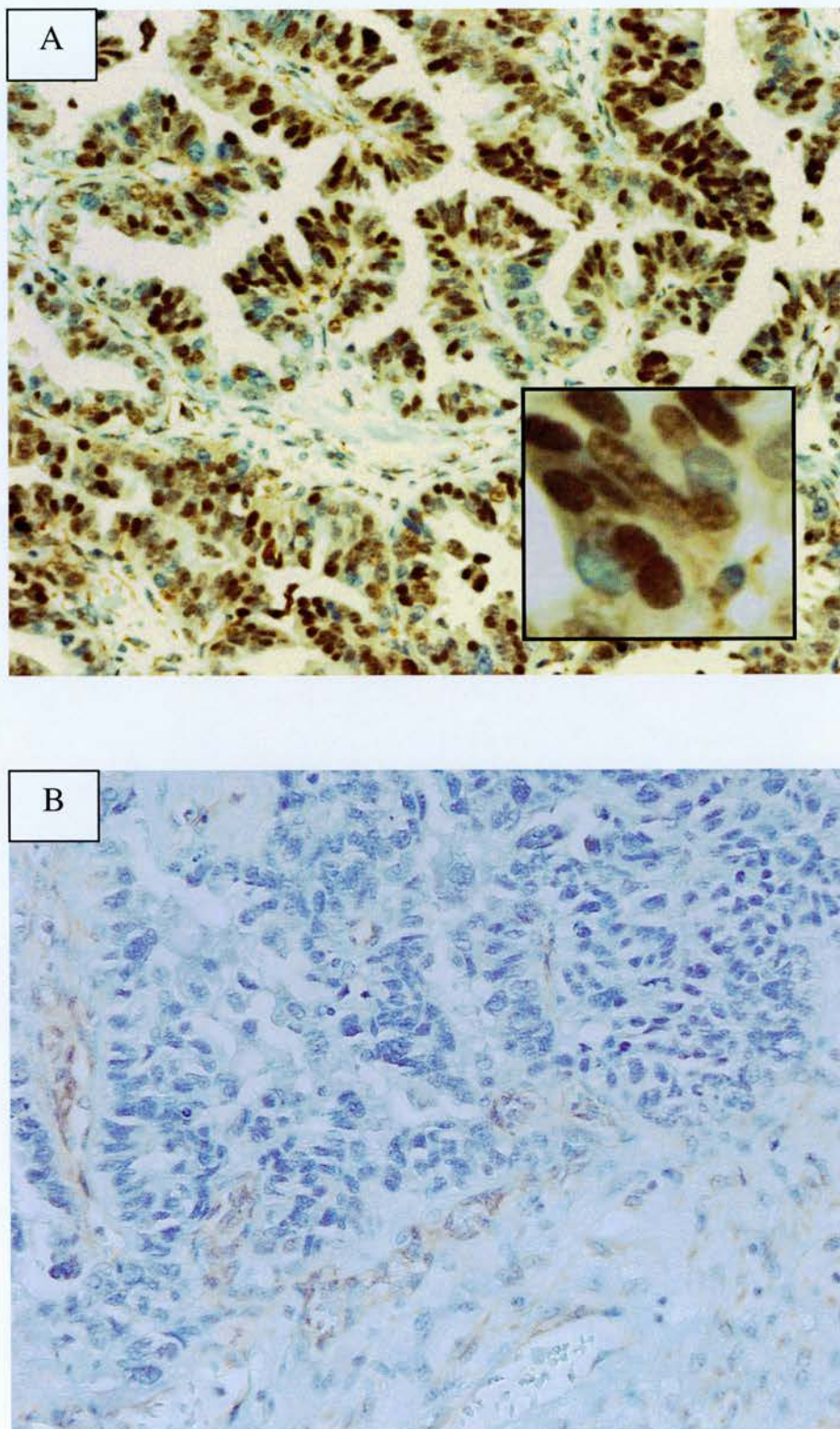
Figure 4.5 (A) Marked overexpression of cyclin E in a well to moderately differentiated serous ovarian cancer. Insert represents area at x400 magnification (B) Absence of cyclin E expression in a poorly differentiated serous ovarian cancer. (x200 magnification).

4.3.6 Clinicopathological and molecular correlates of pRb protein expression

Accumulation of pRb in tumour specimens was considered if >20% of cancer cell nuclei stained positive for pRb protein. The pattern of nuclear staining was intense and homogenous in the invasive tumour. Positive and negative immunohistochemical staining of pRb in tumour tissue is demonstrated in Figure 4.6. Expression of pRb did not correlate with the clinical parameters of stage (p=0.39), grade (p=0.17), volume of residual disease (p=0.18) or presence of ascites (p=0.29).

There was no correlation between the expression of $p21^{Waf1/Cip1}$, $p27^{Kip1}$, cyclin D1, cyclin E, and $p16^{Ink4a}$ and nuclear accumulation of pRb.

Table 4.8   Correlation between pRb expression and clinicopathological and molecular parameters

				pRb (n=134)		
				Positive > 20%	Negative ≤ 20%	p-value
Stage	I	10	(90%)	9 (90%)	1	
(n=134)	II	4	(50%)	2 (50%)	2	0.39
	III	102	(78%)	80 (78%)	22	
	IV	18	(83%)	15 (83%)	3	
Grade	1	7	(5%)	7 (100%)	0	
(n=134)	2	64	(48%)	47 (73%)	17	0.17
	3	63	(47%)	52 (82%)	11	
Ascites	No	45	(34%)	36 (80%)	9	0.29
(n=133)	Yes	88	(66%)	63 (72%)	25	
RD	≤ 2 cm	103	(77%)	74 (70%)	29	0.18
(n=134)	> 2 cm	31	(23%)	26 (70%)	5	

Table 4.8   Continued.

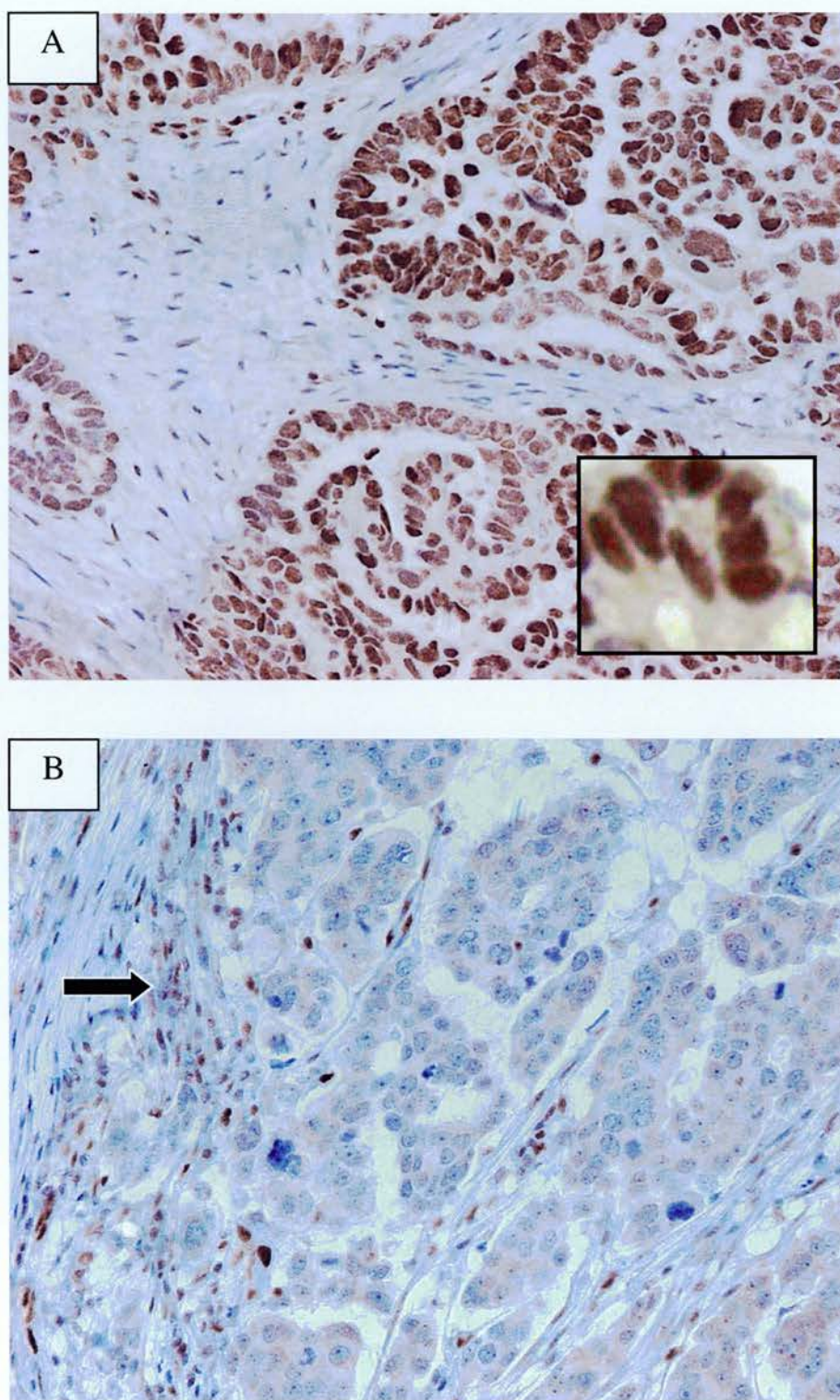|	pRb (n=134)		
	Positive > 20%	Negative ≤ 20%	p-value
p53 (n=134)			
Positive > 10%	56	20	0.77
Negative ≤ 10%	44	14	
p21$^{Waf1/Clp1}$ (n=134)			
Positive > 10%	21	10	0.31
Negative ≤ 10%	79	24	
p27^{Klp1} (n=134)			
Positive > 65%	32	14	0.33
Negative ≤ 65%	68	20	
p16^{Ink4a} (n=134)			
Positive > 15%	46	18	0.48
Negative ≤ 15%	54	16	
cyclinD1 (n=134)			
Positive > 10%	20	5	0.49
Negative ≤ 10%	80	29	
cyclinE (n=134)			
Positive > 10%	58	22	0.49
Negative ≤ 10%	42	12	

Figure 4.6 (A) pRb positive tumour nuclei in a well differentiated serous ovarian cancer. Insert represents area at x400 magnification. (B) pRb negative tumour nuclei in a moderately differentiated serous ovarian cancer. Arrow indicates pRb staining in lymphocytes surrounding tumour cells. (x200 magnification).

4.3.7 Clinicopathological and molecular correlates of p16^{Ink4a} protein expression

High accumulation of p16^{Ink4a} in tumour specimens was considered if >15% of cancer cell nuclei stained positive for p16^{Ink4a} protein. The pattern of nuclear staining was homogenous in the invasive tumour. Positive and negative immunohistochemical staining of p16^{Ink4a} in tumour tissue is demonstrated in Figures 4.7. Expression of p16^{Ink4a} did not correlate with the clinical parameters of stage (p=0.28), grade (p=0.27), volume of residual disease (p=0.46) or presence of ascites (p=0.54)

As mentioned previously there was a significant negative association between the expression of p53 and p16^{Ink4a} (p=0.004). There was no correlation between the expression of p21$^{Waf1/Cip1}$, p27^{Kip1}, pRb, cyclin D1 and cyclin E, and nuclear accumulation of p16^{Ink4a}. The results are shown in Table 4.9.

Table 4.9   Correlation between p16^{Ink4a} expression and clinicopathological and molecular parameters

p16^{Ink4a} (n=134)					
		Number	Positive > 15%	Negative ≤ 15%	p-value
Stage (n=134)	I	10 (8%)	7 (70%)	3	
	II	4 (3%)	3 (75%)	1	0.28
	III	102 (76%)	47 (46%)	55	
	IV	18 (13%)	7 (39%)	11	
Grade (n=134)	1	7 (5%)	5 (71%)	2	
	2	64 (48%)	27 (42%)	37	0.27
	3	63 (47%)	32 (51%)	31	
Ascites (n=133)	No	45 (34%)	23 (51%)	22	0.54
	Yes	88 (66%)	48 (54%)	40	
RD (n=134)	≤ 2 cm	103 (77%)	51 (50%)	52	0.46
	> 2 cm	31 (23%)	13 (42%)	18	

Table 4.9   Continued.

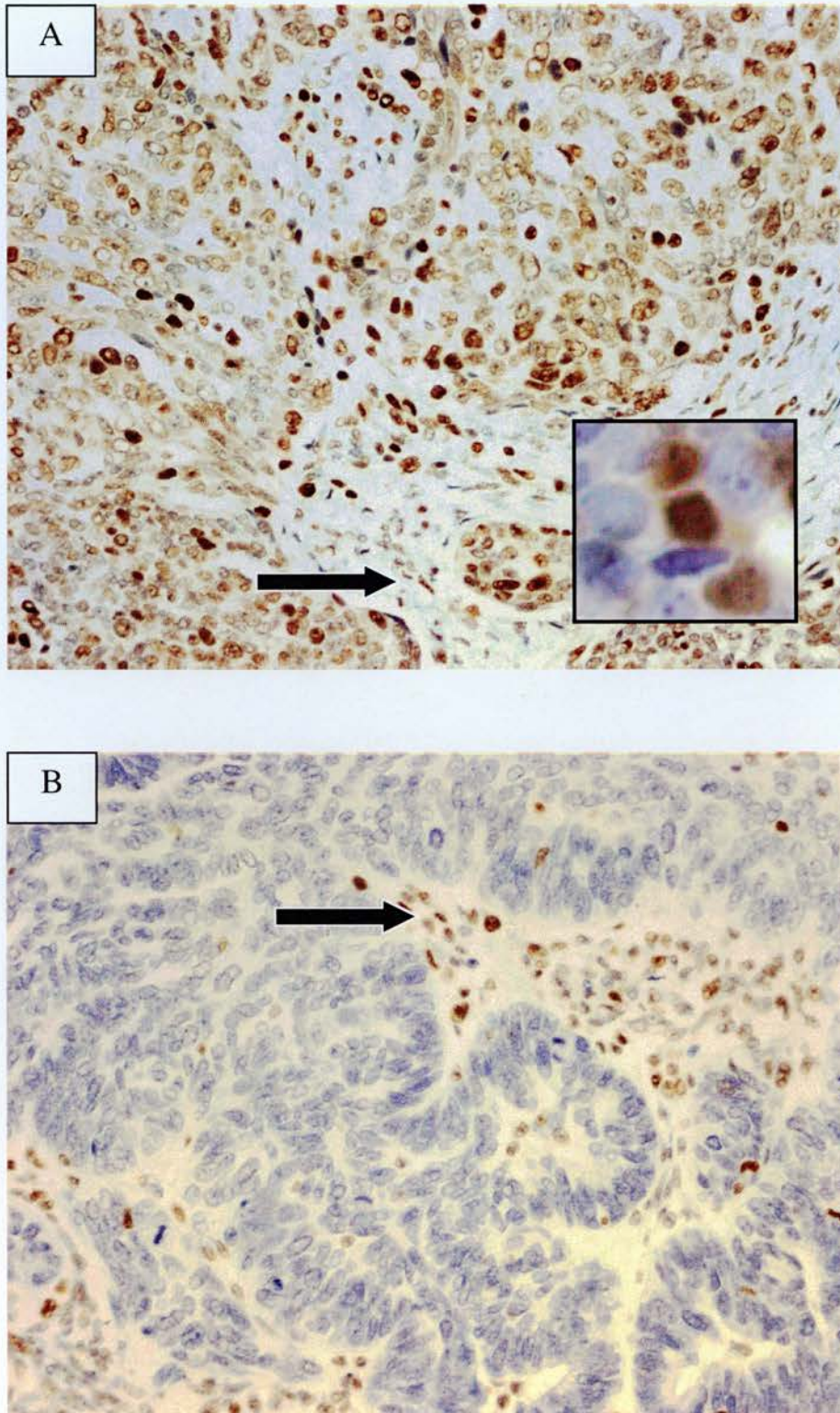| p16^{Ink4a} (n=134)			
	Positive > 15%	Negative ≤ 15%	p-value
p53 (n=134)			
Positive > 10%	28	48	**0.004**
Negative ≤ 10%	36	22	
p21$^{Waf1/Cip1}$ (n=134)			
Positive > 10%	16	15	0.62
Negative ≤ 10%	48	55	
p27^{Kip1} (n=134)			
Positive > 65%	23	23	0.71
Negative ≤ 65%	41	47	
Cyclin D1 (n=134)			
Positive > 10%	9	16	0.19
Negative ≤ 10%	55	54	
Cyclin E (n=134)			
Positive > 10%	41	39	0.33
Negative ≤ 10%	23	31	
pRb (n=134)			
Positive > 20%	46	54	0.48
Negative ≤ 20%	18	16	

Figure 4.7 (A) High p16^{Ink4a} expression in a poorly differentiated serous ovarian cancer. Insert represents area at x400 magnification. (B) Absent p16^{Ink4a} expression in a poorly differentiated serous ovarian cancer. (x200 magnification). Arrows indicate stromal cells displaying expression of p16^{Ink4a} and used as an internal control

4.4 Association between cell cycle gene expression, disease-specific survival and progression-free interval

On univariate Cox regression analysis, molecular markers predictive of reduced DSS included, overexpression of p53 (p=0.02), loss of p21$^{Wafl/Cip1}$ (0.02), increased levels of cyclin D1 (p=0.03) and reduced p27^{Kip1} (0.05). Overexpression of p53 (0.004), loss of p21$^{Wafl/Cip1}$ (p=0.01) and reduced p27^{Kip1} expression (p=0.05) were also predictive of a shorter PFI. Although, a significant association was demonstrated between the expression of p21$^{Wafl/Cip1}$ and p53 (p=0.006), not all p53 negative tumours demonstrated an increase in p21$^{Wafl/Cip1}$ levels. When various combinations of p53 and p21$^{Wafl/Cip1}$ expression were analysed, patients whose tumours demonstrated overexpression of p53, and loss of p21$^{Wafl/Cip1}$ had a significantly shorter DSS and PFI than patients with other combinations of these two molecules (p=0.0008 and p=0.0001 respectively). These data are shown in Table 4.10. Kaplan-Meier curves and log-rank p-value of DSS and PFI stratified according to p53 and p21$^{Wafl/Cip1}$ status are shown in Figures 4.8 and 4.9 respectively.

Table 4.10   Univariate analysis of the relationships between aberrant cell cycle
gene expression and disease-specific survival and progression-free
interval.

Clinical parameter	DSS❖			5-year PFI✳		
	Relative risk	95% CI	p-value	Relative risk	95% CI	p-value
p53 expression ≤10% vs > 10%	1.62	1.05 – 2.50	**0.02**	1.76	1.20 – 2.61	**0.004**
p21$^{Waf1/Cip1}$ **expression** ≤10% vs > 10%	1.90	1.09 – 3.32	**0.02**	1.80	1.13 – 3.0	**0.01**
p27^{Kip1}**expression** ≤65% vs > 65%	1.60	1.0-2.56	**0.05**	1.52	1.0 – 2.30	**0.05**
p16^{Ink4a}**expression** ≤15% vs > 15%	1.20	0.79 – 1.83	0.40	1.29	0.89 – 1.88	0.18
Cyclin D1 expression ≤10% vs > 10%	1.72	1.06 – 2.78	**0.03**	1.39	0.88 – 2.21	0.14
Cyclin E expression ≤10% vs > 10%	1.32	0.86 – 2.05	0.21	1.0	0.69 – 1.47	0.98
pRb expression ≤20% vs > 20%	1.06	0.64 – 1.73	0.83	0.89	0.57 – 1.38	0.61
p53>10% and p21$^{Waf1/Cip1}$**<10%**	2.08	1.36 – 3.20	**0.0008**	2.10	1.43 – 3.09	**0.0001**

❖ Disease-specific survival was calculated from date of primary laparotomy to the date of
disease-specific death or date of last follow up.
✳ 5-year disease-free interval was calculated from the date of primary laparotomy to date
of first reccurrence. Progressive disease as defined às partial or no response to
therapeutic intervention. Data census was at 5 years.
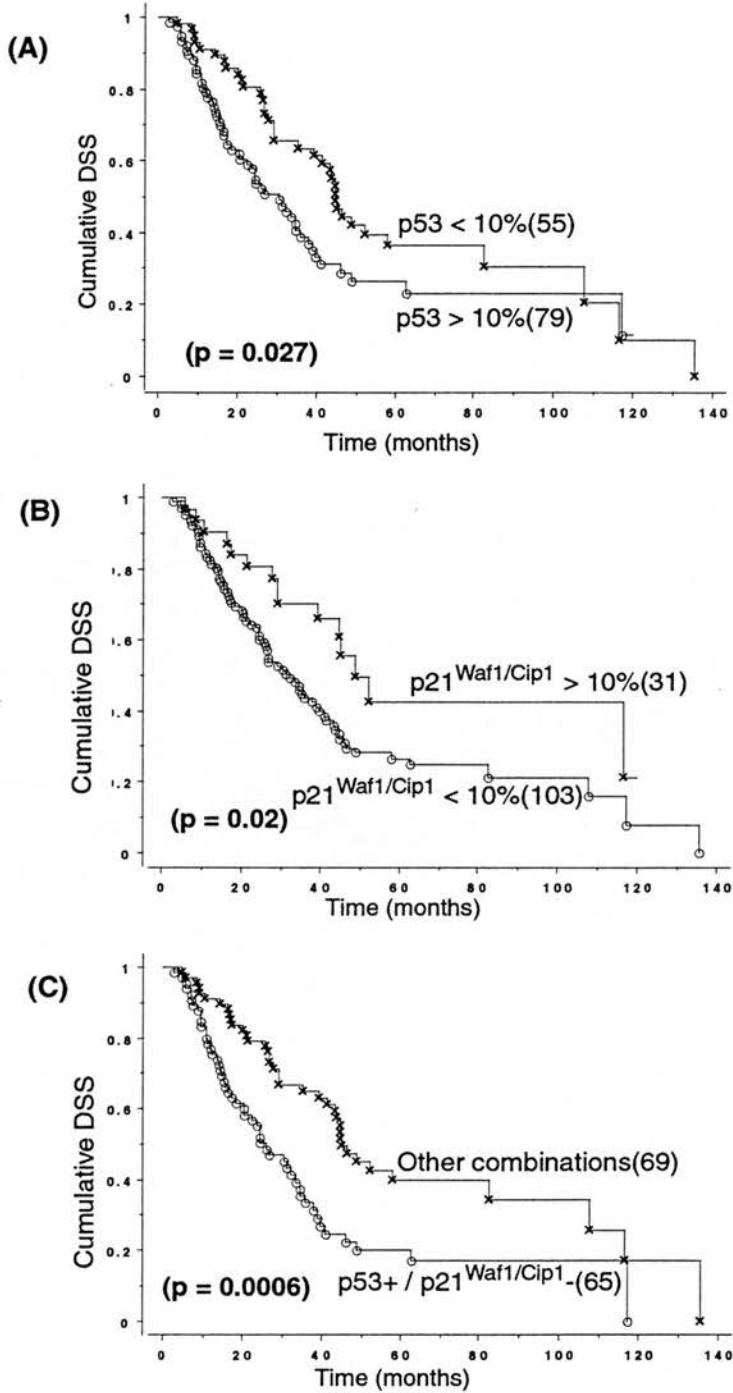
Figure 4.8 Disease-specific survival (DSS) of patients with ovarian cancer according to p53 and p21[Waf1/Cip1] status. (A) Kaplan-Meier curve of DSS according to p53 expression > 10% or < 10%. (B) Kaplan-Meier curve of DSS according to p21[Waf1/Cip1] expression >10% or < 10%. (C) Kaplan-Meier curve of DSS comparing patients with combined expression of the two antigens: (○) p53+(>10%)/p21[Waf1/Cip1]-(<10%), (x) all other combinations of p53 and p21 Waf1/Cip1 expression.

Figure 4.9   5-year progression-free interval (PFI) of patients with ovarian cancer according to p53 and p21$^{Waf1/Cip1}$ status. (A) Kaplan-Meier curve of PFI according to p53 expression > 10% or < 10%. (B) Kaplan-Meier curve of PFI according to p21$^{Waf1/Cip1}$ expression >10% or < 10%. (C) Kaplan-Meier curve of PFI comparing patients with combined expression of the two antigens: (o) p53+(>10%)/p21$^{Waf1/Cip1}$-(<10%), (x) all other combinations of p53 and p21Waf1/Cip1 expression.

The staining pattern of p53 across the tumour specimens revealed a bimodal distribution. p53 expression of <5% was observed in 53 (40%) patients, >50% in 55 (41%) patients with the remainder (19%) being equally distributed between 5-50%. Only 2 (8%) patients that had p53 expression of >5% but <50% were alive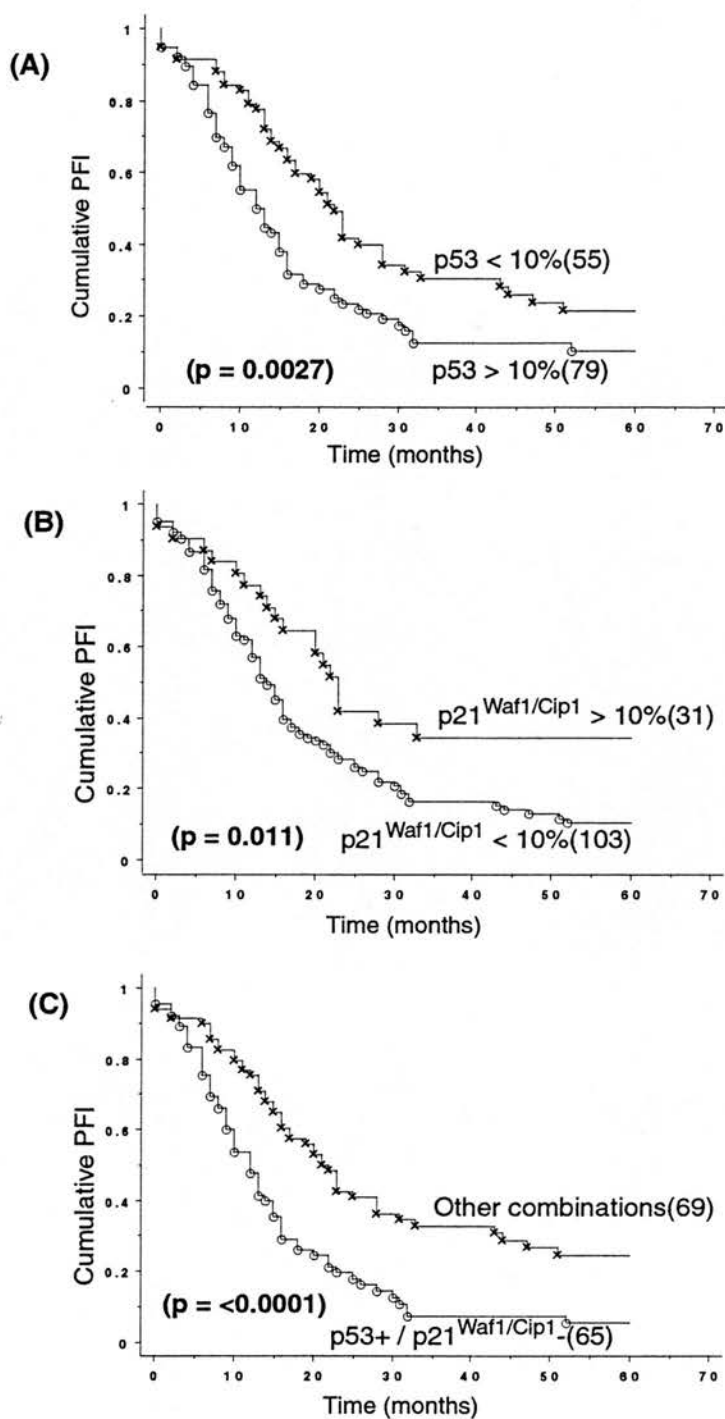 at 5 years, in contrast to 23 (43%) in whom p53 expression was <5% (p<0.0001). Interestingly, 5-year survival rates of patients with p53 expression >50% was closer to the latter group, at 36%. A similar association between expression of p53 and PFI was also demonstrated (p<0.0001). The distribution of p53 in the tumours cohort is shown in Figure 4.10. Kaplan-Meier survival curves and log-rank p-value of DSS and PFI are illustrated in Figure 4.11.
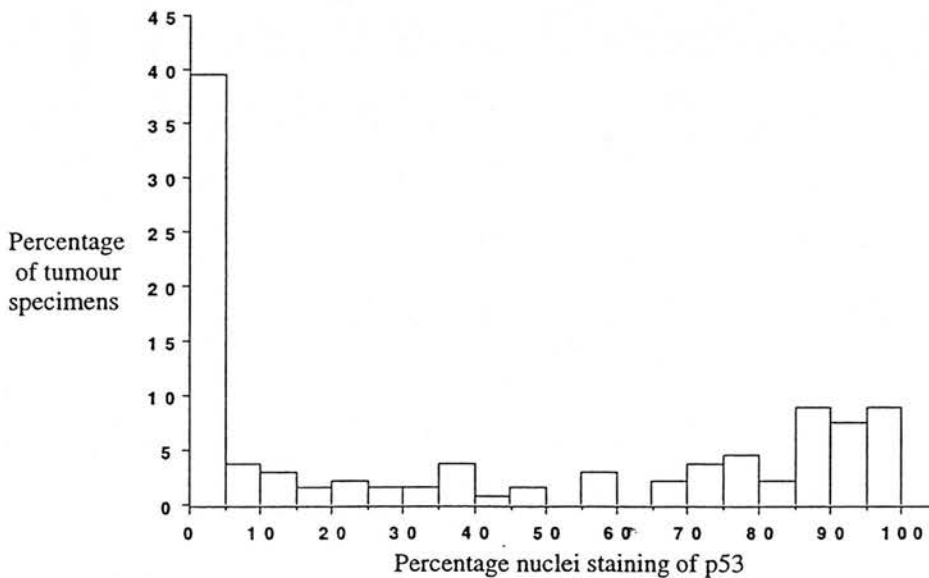
Figure 4.10

This histogram illustrates the expression of p53 in serous epithelial ovarian tumours. The x-axis represents the range of nuclear staining for p53 across the tumour cohort in increments of 5%. The y-axis represents the percentage of tumours displaying p53 experession at a given level.

A **Disease-Specific Survival**

p53<5%

p53>50%

p = <0.0001

p53>5%<50%

Probability of DSS

Time (months)

B **Progression-Free Interval**

p53<5%

p53>50%

p = <0.0001

p53>5%<50%

Probability of PFI

Time (months)

Figure 4.11 Kaplan-Meier curve for 5-year DSS (A) and PFI (B)
according to p53 stratification. Patients were assigned
3 subgroups according to their percentage of p53
nuclear staining. (x) p53 < 5% (n=53 (40%)),
(•) p53>5%<50% (n=26 (19%)), (▲)p53>50% (n=55 (41%))

4.5   Multivariate Analysis

The results of multivariate Cox-regression analyses for disease-specific survival and progression-free interval are shown in Table 4.11a and 4.11b. In this study, the age of patients at the time of diagnosis and pre-operative serum CA125 levels were not significant in determining disease outcome on univariate analysis and were consequently not included in the multivariate models. In addition, tumour grade and clinical stage were also not incorporated in the multivariate analysis. The number of well differentiated tumours was small in this cohort, and no survival advantage for moderately differentiated tumours could be demonstrated in comparison to the poorly differentiated ones. Moreover, the expression of several molecular markers were found to significantly co-segregate wit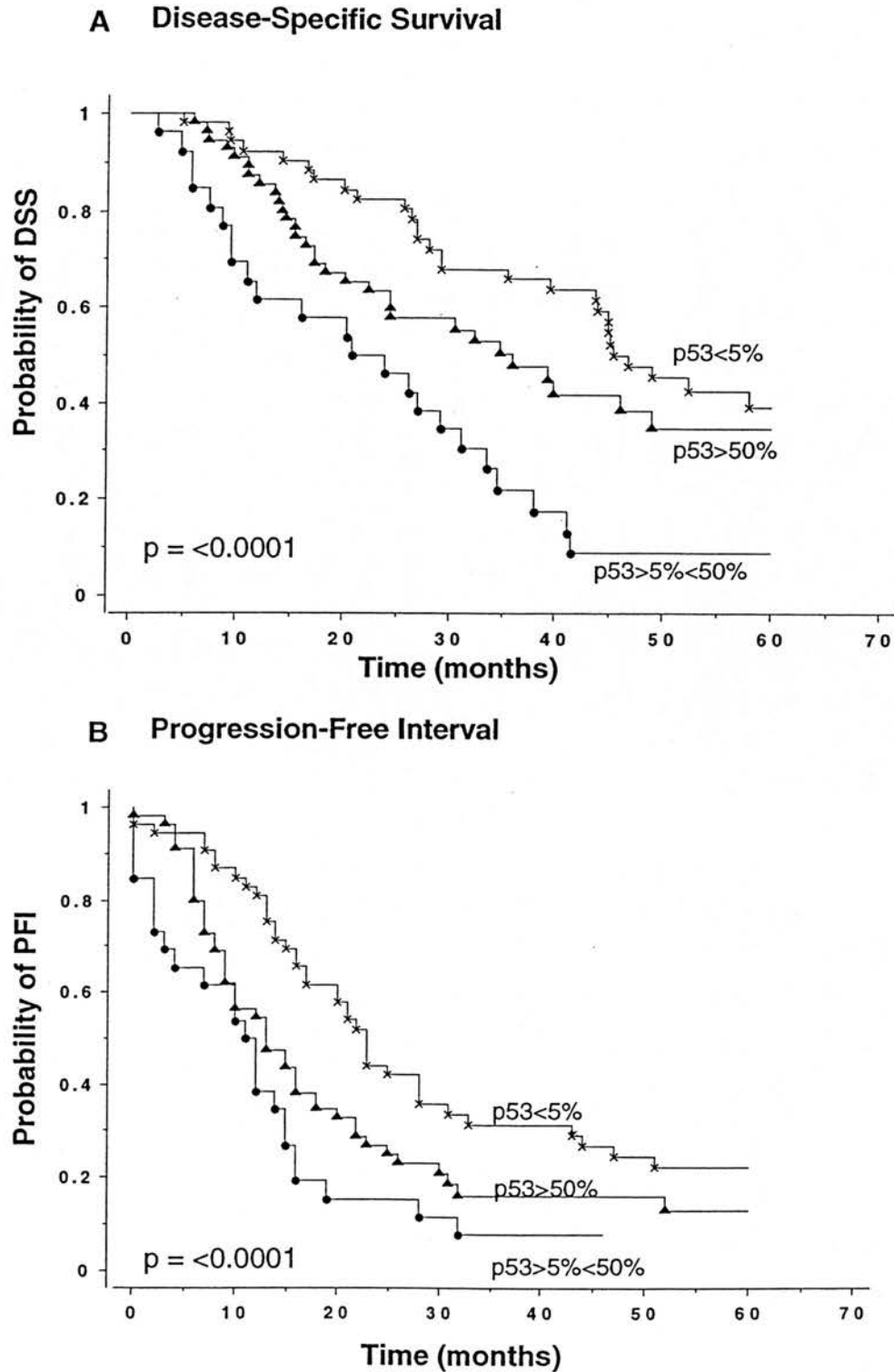h degree of tumour differentiation in particular p21$^{Wafl/Cip1}$ (p=0.006). Similarly, stage was not included either, due to the relatively small number of FIGO stage I and II cancers. In addition, larger volume of post-operative RD and presence of ascites was significantly associated with advanced tumour stage (data not shown).

The volume of post-operative residual disease remained the most important determinant of DSS and PFI. Cyclin D1 overexpression and a combination of p53 overexpression and loss of p21$^{Wafl/Cip1}$ were also independent predictors of DSS, with the latter retaining its independent role in determining PFI. The presence of ascites and performance status of patients became insignificant in both multivariate models.

Table 4.11a Multivariate Cox regression analysis of overall DSS of patients with serous EOC.

Disease-specific survival			
Clinical Parameter	**Relative risk**	**95% confidence interval**	**p-value**
Residual disease ≤2cm vs > 2cm	2.91	1.68 – 5.03	**0.0001**
Performance Status 0 vs 1,2	1.55	0.96 – 2.52	0.073
Ascites Presence vs Absent	1.29	0.75 – 2.22	0.35
p53>10% and p21<10%	2.35	1.44 – 3.83	**0.0006**
cyclin D1 ≤10% vs 10%	1.75	1.03 – 2.96	**0.038**

Disease-specific survival was calculated from date of primary laparotomy to the date of disease-specific death or date of last follow up.

Table 4.11b Multivariate Cox regression analysis of 5-year PFI of patients with serous EOC.

5-year Progression-free Interval			
Clinical Parameter	**Relative risk**	**95% confidence interval**	**p-value**
Residual disease ≤2cm vs > 2cm	2.76	1.64 – 4.64	**0.0001**
Performance Status 0 vs 1,2	1.17	0.75 – 1.82	0.49
Ascites Present vs Absent	1.45	0.91 – 2.30	0.12
p53>10% and p21<10%	2.20	1.44 – 3.37	**0.0003**

5-year progression-free interval was calculated from date of primary laparotomy to the date of first relapse or progression.

Chapter Five   Discussion

5.1   Introduction

Ovarian cancer is the leading cause of death from gynaecological malignancies in the western world.   The course of this disease is dependent upon the clinical characteristics of the affected individuals and the pathological features of their cancer.   Due to the insidious nature of this disease, patients often present with late stage cancer in whom prognosis is poor.   Other clinicopathological prognosticators of disease outcome include the degree of tumour differentiation, presence of ascitis, performance status of patients and volume of residual disease.   With an increasing understanding of genetic events involved in the neoplastic process, clinicians and scientists are hoping to add to the clinicopathological determinants of ovarian cancer progression, by analysing molecular markers thought to be relevant in cancer biology.   In addition to allowing estimates of clinical outcome, the in-vivo molecular snapshots will broaden our understanding of basic cancer biology and may guide future treatment based on a particular subset of genetic alterations.

Cancer *per se* is thought to arise from the step-wise accumulation of genetic alterations that result in uncontrolled cellular proliferation and loss of differentiated functions.   In epithelial ovarian cells these abnormalities mainly accumulate as a consequence of rapidly dividing cells and possible DNA damage during ovulation.   Dysregulation of cell cycle control has been implicated in the pathogenesis of most human cancers.   A precise balance between cyclins and CDKI's is critical to G_1/S phase transition, and abnormal expression of these molecules is observed frequently in ovarian cancer.   Since 1994, several studies have been published that evaluate the impact of these molecular markers on the survival of ovarian cancer patients.   Brief summaries of findings from several of these reports are detailed in Tables 2.2 – 2.4. However, despite the accumulating number of publications there remain problems in interpreting these results into meaningful clinical information.   These problems include:

- Small sample size in many studies which limits statistical power and subgroup analyses.

- The inclusion of several subtypes of epithelial ovarian cancers as a single entity in the final analyses of overall and progression-free survival of patients, in the majority of reports. Some reports even include ovarian tumours of low malignant potential (borderline) in their overall analyses.

- Details of statistical analysis are often obscure, in particular why certain factors were removed or excluded from multivariate models. In addition, only results from univariate analyses are reported in a significant number of studies.

- Non-uniform patient and disease populations. A few but significant numbers of studies have reported "valuable insights into ovarian tumour biology" by evaluating the expression of candidate genes across benign, borderline and invasive ovarian tumours as a progression model. There is, however, no clinicopathological evidence to suggest that such a progression model is valid for ovarian cancer, and to date no pre-invasive model for development of EOC has been identified that is comparable to that reported in other cancers, such as cervical (46) and colorectal cancer (127).

In the present study, the expression of genes critical to G1/S phase transition were examined in a series of one type of EOC, namely serous cystadenocarcinomas. The genes selected for this study are important in two pathways (cyclin D1/CDK4/p16^{Ink4a} and cyclin E/CDK2/p27^{Kip1}) which are influenced by p53 and p21$^{Waf1/Cip1}$, and ultimately converge on pRb (see Figure 2.1). Dysregulation of these pathways is implicated in the pathogenesis of several human cancers but has been studied to a far lesser extent in ovarian cancer with conflicting results. Abnormal expression of these genes was analysed in relation to tumour characteristics and disease outcome. The cohort was selected from a consecutive series of patients treated for serous EOC at a tertiary referral gynaecology oncology centre. This is reflected in the higher proportion of advanced FIGO stage III/IV tumours, than in the published literature (8). Nevertheless, examination of traditional clinicopathological variables confirmed that the cohort behaves in accordance with published series of

epithelial ovarian cancers with regard to disease-specific and progression-free survival of patients (60-67).

5.2   p53 and p21$^{Waf1/Cip1}$ expression in serous epithelial ovarian cancer

p53 is a transcriptional regulator that is activated by a wide range of cellular stresses, and is involved in cell cycle regulation, apoptosis and angiogenesis (282). These stresses include DNA damage, hypoxia and redox stress. Inactivation of this tumour suppressor gene is implicated in tumourigenesis of several human cancers (283), and accumulation of mutated p53 is an adverse prognostic indicator in breast (284), lung (285) and colorectal cancer (286). Gene mutations and/or overexpression of p53 have been detected in 30 – 80% of epithelial ovarian tumours (234, 241, 246, 254). A significant association between p53 immunostaining and p53 gene mutation in ovarian cancer has also been reported in a number of studies (234, 246, 287, 288) in accordance with the view that most p53 mutations lead to stabilisation of the protein. In the largest series published to date Wen et al., (1999), examined p53 gene mutation and expression in 105 epithelial ovarian tumours. Eighty-two percent of the 60 tumours displaying DNA sequence alterations were due to missense mutations and accumulation of p53 protein was observed in 92% of these cancers. The remainder included nonsense and frameshift mutations, 50% of which displayed p53 overexpression. A small number of false positives were also observed but nevertheless, a strong association between p53 mutation and expression was reported (234). While it is likely that direct sequencing is the most reliable method for determining p53 mutations, immunohistochemistry offers a practical tool for analysing p53 protein accumulation in clinical specimens.

In this study less than 5% nuclear accumulation of p53 was observed in 53 specimens, greater than 50% accumulation in 55 tumours with the remainder (n = 26) demonstrating intermediate expression. No cytoplasmic staining of p53 was observed in any tumour specimens. Less than 10% of patients with intermediate expression of p53, i.e. between 5% and 50%, were alive at 5 years which was a

significantly poorer outcome than the other 2 groups. A similar association between the pattern of p53 expression and progression-free interval was also demonstrated. The reason for this is unclear, although there are a number of potential explanations that require further investigations. It is possible that tumours overexpressing wild type p53 may exhibit some degree of immunostaining for p53 protein. In normal cells, p53 is present at extremely low levels because the protein is rapidly degraded following synthesis (289, 290). Levels of p53 increase rapidly in response to cellular stresses, and with dramatic decrease in degradation through post-translational modifications, and added stability by its interactions with other intracellular proteins, positive immunostaining may result (231, 236, 237). For example mdm-2 is a regulator of the homeostatic and functional attributes of p53. In addition to being a transcriptional target of p53, mdm-2 forms a complex with p53 that negatively influences p53's transcriptional function in-vivo. Moreover, mdm-2 acts as a nuclear-cytoplasmic shuttle of p53 and thereby playing a significant role in the mdm-2 mediated degradation of p53 by the ubiquitin dependent system. Secondly, certain types of *p53* mutations may result in very high levels of p53 protein that may have some residual activity in regulating cellular functions. For example, a few studies have found a better response to chemotherapy in laryngeal and bladder cancers that display marked overexpression of p53 (291, 292). Thirdly, *p53* point mutations resulting in a stop codon, frameshift or nonsense mutations may result in altered or truncated proteins that are detected using routine sequence analysis but not detected by immunohistochemistry (293). Ovarian tumours with *p53* gene mutations resulting in truncated proteins have been shown to develop distant metastases more rapidly than tumours with missense or no mutations, and consequently survival in these patients is significantly shorter (294). More comprehensive mutation and functional studies are required to decipher the role of mutant and wt p53 in ovarian carcinogenesis.

The prognostic value of p53 accumulation as a marker of poor outcome in ovarian cancer is controversial. A few studies report p53 overexpression as an adverse factor of clinical outcome in ovarian cancer (241-245, 295) whereas several have failed to confirm such findings (246-248, 256). In addition, most studies reporting

significant results in univariate analysis fail to demonstrate an independent role of p53 overexpression in multivariate analysis, when adjusted for traditional clinicopathological characteristics of disease outcome. In the study, p53 overexpression was found to be an independent marker of shorter disease-specific and progression-free survival when considered with residual disease, presence of ascites, performance status and cyclin D1 expression. However, the prognostic power of p53 strengthened significantly when analysed in combination with p21$^{\text{Waf1/Cip1}}$ expression.

Historically, the expression of p21$^{\text{Waf1/Cip1}}$ has been considered a surrogate marker of p53 function. Levels of p21$^{\text{Waf1/Cip1}}$ are predominantly regulated at the transcription level by p53-dependent mechanisms, although there is increasing evidence to suggest p53-independent pathways of p21$^{\text{Waf1/Cip1}}$ induction also exist. For example, in gastric adenocarcinomas TGF-beta1 may be involved in the activation of p21$^{\text{Waf1/Cip1}}$, whereas in prostate cancer androgens may directly activate transcription (298). Altered degradation and interaction with other cellular molecules may also influence levels of p21$^{\text{Waf1/Cip1}}$. Studies involving fibroblasts from *p53* knock out mice (211) and ovarian and breast cancer cell lines (296, 297) with *p53* mutations, show that up-regulation of p21$^{\text{Waf1/Cip1}}$ can occur independent of *p53* status. In this present study, a significant negative correlation was found between the expression of p21$^{\text{Waf1/Cip1}}$ and p53 accumulation. p21$^{\text{Waf1/Cip1}}$ loss in the presence of p53 overexpression occurred in 65 cancer specimens. However, there were other tumours (n=11) that demonstrated a concurrent increase in the expression of both p53 and p21$^{\text{Waf1/Cip1}}$ suggesting that at least in these tumours, p53-independent mechanisms may exist that upregulate p21$^{\text{Waf1/Cip1}}$ levels. These results are consistent with findings in other studies of ovarian cancer (244, 258). The mechanisms of p53-independent regulation of p21$^{\text{Waf1/Cip1}}$ in ovarian cancer is unclear and warrants further research.

To investigate further the relationship between p53 and p21$^{\text{Waf1/Cip1}}$, the impact of aberrant expression of these two molecules on clinical outcome of patients was examined. A limited number of studies have examined the prognostic value of p21$^{\text{Waf1/Cip1}}$ in EOC, and /or its association with p53 (244, 245, 255, 256). The role

of p53 as a prognostic marker in our study has been discussed previously. For p21$^{Waf1/Cip1}$, univariate analysis showed that loss of this protein in serous epithelial ovarian cancers is significantly associated with poor survival and a shorter progression-free interval. These results in part, support findings by other investigators (244, 255), although this study reports for the first time an association between aberrant p21$^{Waf1/Cip1}$ expression and a shorter interval to disease progression. However, an independent role for p21$^{Waf1/Cip1}$ in predicting disease outcome when considered with other molecular and clinical variables was not found. This was mainly due to the incorporation of p53 expression in multivariate models. Bivariate analysis of survival with p53 alone showed that the significance of aberrant p21$^{Waf1/Cip1}$ expression was not retained, which may suggest levels of p21$^{Waf1/Cip1}$ are predominantly regulated by p53-dependent mechanisms in our cohort of serous ovarian tumours. However, despite this and the negative correlation between the expression of these two proteins in this study, the prognostic relevance of combining p53 and p21$^{Waf1/Cip1}$ expression in predicting clinical outcome was analysed. It was found that loss of p21$^{Waf1/Cip1}$ in conjunction with p53 overexpression was a stronger predictor of reduced survival and progression-free interval, than other combinations of expression or the aberrant expression of either molecule alone. This would suggest the expression of p21$^{Waf1/Cip1}$ is not simply a surrogate marker of p53 function, and in part supports our previous observations of possible p53-independent mechanisms of p21$^{Waf1/Cip1}$ induction. These results are consistent with findings in one other study of ovarian cancer (244). Anttila *et al.*, (1999) reported a significant co-segregation between p53 and p21$^{Waf1/Cip1}$ in 305 tumours of ovarian origin, and demonstrated increased power in predicting clinical outcome when combining the abnormal expression of both molecules than either alone. However their tumour cohort comprised 5 different subtypes of ovarian cancers with 53 being classified as miscellaneous (244).

p21$^{Waf1/Cip1}$ is involved in growth arrest and terminal differentiation in different cell types (203, 211). Accumulation of p21$^{Waf1/Cip1}$ correlates with well differentiated tumours of breast (298) and oral cancer (206). In agreement with these observations, the results presented support a significant correlation of elevated p21$^{Waf1/Cip1}$ protein

in well differentiated serous ovarian tumours with marked loss of p21$^{Waf1/Cip1}$ expression in moderately differentiated and poorly differentiated cancers. A dramatic loss of p21$^{Waf1/Cip1}$ expression was observed between grade I and II tumours and a smaller difference between grade II and III cancers. However, in this study there were relatively few well differentiated tumours and a larger series of serous epithelial ovarian cancers will be required to confirm this result. The expression of p21$^{Waf1/Cip1}$ did not correlate with other clinicopathological features of malignancy, which is consistent with results from other studies in ovarian cancer (244, 245, 256)

5.3 pRb / cyclin D1 / p16^{Ink4a} pathway in serous epithelial ovarian cancer

Cyclin D1 is a positive regulator of G$_1$ progression, and is a putative oncogene whose aberrant expression has been implicated in the pathogenesis of several types of cancer. Cyclin D1 overexpression with or without gene amplification has been identified as an adverse prognostic biomarker in breast (299), tongue (261), lung (264), pancreatic (265) and gall bladder cancers (300). Amplification or mutation of the *CCND1* gene, encoding cyclin D1, is rarely observed in ovarian cancer, although increased levels of mRNA and protein overexpression have been reported to occur in 14-95% of invasive tumours (155, 156, 266, 267). External mitogenic signalling and regulation by other proteins integral to cell cycle progression may account for this overexpression. For example, activating protein-1 (AP-1) complexes bind to specific DNA sequences in the regulatory motifs of mitogen-responsive genes, that includes *CCND1*, which leads to cellular proliferation after stimulation by external mitogens such as EGF, TPA or oestrogen (301). In breast cancer, a significant association is demonstrated between cyclin D1 expression and fra-2, a member of the AP-1 family of activating proteins (302). Recent evidence also suggests that levels of TGF-α and EGFR are independent predictors of outcome in head and neck and oesophageal cancers possibly mediated via increased expression of cyclin D1 (303).

This is the first study to report an independent prognostic role of cyclin D1 in determining disease-specific survival of patients with serous epithelial ovarian cancer. Nineteen percent of tumours demonstrated >10% nuclear accumulation of cyclin D1 which is consistent with other reports of cyclin D1 overexpression in ovarian cancer (266). The 5-year survival rate of patients in cyclin D1 positive and negative groups was 9% and 39% respectively. Interestingly, there was no apparent difference in survival between the 2 groups at 24-month census. Overexpression of cyclin D1 was not associated with a shorter progression-free interval which may explain why no difference was observed in the disease-specific survival of patients at 24-months follow up as already mentioned. In addition, there was a trend towards cyclin D1 nuclear overexpression with increasing grade of tumour although this did not reach significance. Overexpression of cyclin D1 was absent in all well differentiated tumours and present in 14% of moderately differentiated and 23% of poorly differentiated tumours.

The role of cyclin D1 in ovarian cancer has not been extensively studied. Barbieri *et al.*, (1999) are the only authors to report a significant association between cyclin D1 expression and disease outcome in a small series of epithelial ovarian cancers (267). In brief, the authors investigated the relationship of cyclin D1 overexpression at the mRNA and protein level in a series of 55 benign adenomas, 12 borderline tumours and 37 carcinomas (including 5 recurrences). Cyclin D1 mRNA expression was evaluated using RT-PCR and protein expression by Western blotting. mRNA was detected in 95% of ovarian carcinomas, with 44% exhibiting strong signal intensity. This was also reflected at the protein level. The positive association between cyclin D1 protein overexpression and mRNA overexpression is consistent with other reports in pancreatic (304) and oesophageal tumours (304). Overall survival in the 32 patients with primary ovarian carcinoma investigated with regard to cyclin D1 mRNA overexpression (defined by strong signal intensity), was calculated using the Kaplan-Meier method and log-rank test. However, only 24 of these patients were included for analysis of progression-free survival. The reason for this is unclear. Nevertheless, a significantly shorter progression-free interval was observed in those patients overexpressing cyclin D1. There was no difference in overall survival. The

authors also reported a significant association between cyclin D1 overexpression with serous carcinomas and poorly differentiated tumours. In contrast to this latter finding, earlier studies of cyclin D1 overexpression in ovarian cancer have reported a significant co-segregation with well-differentiated tumours (155, 266, 268). However, in these studies the number of cases investigated is small. In one study, borderline tumours are included with invasive malignancies in the correlation tests (268) and with the large diversity of subtypes of EOC's studied in the remainder, the results are difficult to interpret. Nevertheless, a similar finding has been reported in breast cancer (269). However, other studies have found that cyclin D1 overexpression can inhibit the differentiation of myoblasts (305), and intestinal epithelial cells (306), thus raising the possibility that cyclin D1 overexpression may play a role in the inhibition of tumour cell differentiation in some cell types. In this study we observed a trend but not a significant co-segregation between cyclin D1 overexpression and tumour grade (likely due to the small number of well-differentiated tumours), and a larger study may be warranted to further define the role of cyclin D1 in tumour differentiation in ovarian cancer.

$p16^{Ink4a}$ is a tumour suppressor gene implicated in the pathogenesis of many different cancers. Its protein product plays a role in the cell cycle, cell senescence, anoikis, integrin-mediated cell spreading and angiogenesis (225, 226). Within the G_1 phase of the cell cycle it acts predominantly by inhibiting cyclin D1/CDK4-6 complexes, thereby preventing phosphorylation of pRb (223). Inactivation of $p16^{Ink4a}$, mainly by promoter methylation and homozygous deletions, is frequently observed in human cancer and loss of p16^{Ink4a} is associated with poor clinical outcome in head and neck (261) and oesophageal cancers (229). Although loss of heterozygosity is present in 30-40% of ovarian cancers, inactivating mutation of the remaining allele or promoter methylation is rarely observed (307). In addition, there is no evidence to suggest that aberrant expression of p16^{Ink4a} is associated with clinical outcome in this cancer (153, 263). In this study, there was no association between aberrant p16^{Ink4a} expression with clinicopathological features of malignancy or clinical outcome of patients with serous epithelial ovarian tumours. The *Ink4A/ARF* locus located on chromosome 9 also encodes for a tumour suppressor gene called $p14^{AF}$ ($p19^{ARF}$ in

mice). Transcription of this gene is activated through various means including the E2F family of transcription factors and the resultant protein product converges on the p53 pathway. There is thought to be extensive cross talk between the $p16^{Ink4a}$ / Rb and the $p14^{ARF}$ / p53 pathways. It is therefore possible that levels of $p16^{Ink4a}$ and p53 may be partly dependent, and in this study there was noted to be a significant negative correlation between the expression of $p16^{Ink4a}$ and p53. Of those p53 positive tumours, 37% demonstrated positive $p16^{Ink4a}$ expression, whereas in p53 negative tumours there was positive $p16^{Ink4a}$ in 62% of tumours. Further research is required to fully decipher the function and interactions of $p16^{Ink4a}$.

The Rb gene family encodes a group of related proteins that participate in several aspects of cell cycle regulation and control of gene expression. p107 and p130 have a 30-35% amino acid identity to pRb, that also interact with the E2F family of transcription factors. An important characteristic that distinguishes this family of related proteins is the cell cycle timing of their interactions. pRb acts predominately in the G_1 phase whereas p107 and p130 is thought to act mainly in the S and G_0 phases respectively. However, the role of these Rb related proteins in the p16/cyclin D1/CDK4 control pathway is unclear and is presently being explored. *Rb* gene mutation and loss of pRb have been described in a variety of tumours that include gall bladder (180), breast (181), lung (182), and bladder cancers (308). Loss of pRb in these tumours is predictive of poor clinical outcome and is associated with advanced stage and increasing tumour grade. Phosphorylation and inactivation of pRb by cyclin/CDK complexes in G_1 allows the release of transcription factors that are necessary for cellular progression into S phase, and hence this protein is of paramount importance in cell cycle regulation. However, similar to *$p16^{Ink4a}$*, despite frequent loss of heterozygosity, alterations of the remaining allele and loss of pRb in ovarian cancer is rare. Studies using immunohistochemistry to detect pRb in ovarian cancer have reported that 70-85% of tumours express pRb and that loss of this protein in the small minority of cancer specimens does not appear to co-segregate with clinicopathological features of malignancy (183, 274, 275). The findings presented in this study support the results from previous studies. pRb was present in

79% of tumour specimens and loss was not associated with clinical outcome of patients or other clinicopathological features of malignancy.

5.4   p27^{Kip1} and cyclin E expression in ovarian cancer

A trend for low p27^{Kip1} expression and poor tumour differentiation has been reported in breast (221), colon (309, 310) and bladder carcinoma (311). In addition, experiments involving p27^{Kip1} deficient mice show multiorgan hyperplasia, loss of terminal differentiation and tumourigenesis (215, 217). Further evidence for the role of p27^{Kip1} in cellular differentiation comes from studies involving human colon cancer cell lines (312, 313). Overexpression of p27^{Kip1} results in partial growth inhibition in these cancer cell lines and markedly increases sensitivity to the induction of cell differentiation (312). The current study is the first to report a significant correlation between reduced levels of p27^{Kip1} expression and increasing tumour grade in epithelial ovarian cancer. Fifty-seven percent of well differentiated tumours displayed increased levels of p27^{Kip1}, in contrast to 42% of moderately differentiated and 23% of poorly differentiated cancers. The number of studies examining the expression of p27^{Kip1} in ovarian cancer is limited (257, 259, 266, 314), and the majority have been unable to support a role of p27^{Kip1} in tumour differentiation. The results of this study are consistent with one previous study that demonstrated reduced expression of p27^{Kip1} was significantly associated with increasing grade in a panel of ovarian tumours (266). The expression of p27^{Kip1} and cyclin D1 was examined by immunohistochemistry in 79 ovarian tumours that included 22 benign cystadenomas, 23 borderline tumours and 34 ovarian cancers. A steady decline in the levels of p27^{Kip1} and cyclin D1 were observed from benign to malignant lesions. Of the 34 invasive EOC's, increased expression of p27^{Kip1} and cyclin D1 appeared to significantly co-segregate with well differentiated tumours, although no difference was observed between moderately and poorly differentiated tumours (the result relating to cyclin D1 overexpression and tumour differentiation in this study, is however contradictory to observations in this present cohort). However, with the limited number of specimens and even smaller subgroups of

tumours when stratified for the degree of tumour differentiation (grade 1 (n=17), grade 2 (n=9) and grade 3 (n=8)) the results reported in this study need to be interpreted with caution.

Cyclin E is a late G1 cyclin forming a complex with CDK2 and is a candidate for controlling G1 to S phase transition. High levels of cyclin E correlate with increasing tumour grade in breast (315) and laryngeal cancers (316), although low expression of cyclin E has been observed to associate with increasing tumour grade and lymph node invasion in bladder cancer (311, 317). It has also been shown to be associated with high tumour cell proliferative index in laryngeal cancer, and in these tumours, increased expression was predictive of poorer disease outcome (316). The role of cyclin E in tumour differentiation, cellular proliferation and clinical outcome in ovarian cancer has not been defined. Increased level of cyclin E was associated with increasing tumour grade in our study. The biggest difference was observed between well differentiated and moderately differentiated tumours although a larger study incorporating more well differentiated tumours is required. Gene amplification has been reported in 20% of ovarian tumours, with significantly higher levels of RNA and protein expression (271, 272). In this study overexpression of cyclin E was present in 68% of tumour specimens, which is consistent with the degree of overexpression in other tumour subtypes (316, 318). There was no relationship between the expression of cyclin E and clinical outcome in this current study which supports the reported findings in other studies of ovarian cancer (271, 272).

$p27^{Kip1}$ gene mutation is rare in human cancer (319). Protein levels are mainly regulated by ubiquitin-mediated proteosome degradation, which is targeted by cyclin E/CDK2 phosphorylation of $p27^{Kip1}$ (320). Therefore it is likely that reduced $p27^{Kip1}$ and high cyclin E expression may be associated with cancer progression and unfavourable prognosis. This relationship of aberrant $p27^{Kip1}$ and cyclin E expression is associated with increasing tumour grade and mortality in breast (315) and bladder cancer (311) and malignant lymphomas (321). In this current study, reduced $p27^{Kip1}$ and high cyclin E expression were associated with increasing tumour grade, although there was no significant correlation between expression of these two

molecules. In addition, aberrant expression of p27^{Kip1} but not cyclin E, was associated with unfavourable prognosis of patients. Decreased expression of p27^{Kip1} alone has been identified as an adverse prognostic factor in many tumour types including colon (221, 222), and prostate carcinoma (220). In ovarian cancer, only one study has supported an independent prognostic role of p27^{Kip1} in determining the clinical outcome of patients (259). In that study 33 short-term survivors (<2 years) had significantly reduced p27^{Kip1} levels in comparison to 33 long-term survivors (>5years). The study was limited to FIGO stage II and III tumours and patients surviving between 2 and 5 years were excluded. Positive p27^{Kip1} was considered if >50% tumour nuclei stained positive. A further study of 185 patients reported a trend (which did not reach significance) of reduced overall survival in a small subgroup of patients (n=11) whose ovarian tumours completed lacked p27^{Kip1} expression (257). Due to the small number of cases that lacked p27^{Kip1} expression, all further analyses was considered with p27^{Kip1} expression of >50% as positive staining, and subsequently no correlation to clinicopathological parameters or clinical outcome was reported. In this current study it was observed that reduced p27^{Kip1} expression correlated with poor clinical outcome (shorter DSS and PFI) in univariate analysis, which did not retain significance as an independent prognostic factor when adjusted for other clinicopathological and molecular variables. There are several possible explanations for this. Firstly, a significant correlation was observed between p27^{Kip1} and p21$^{Waf1/Cip1}$ expression in tumour specimens. This finding is consistent with reports from other studies in ovarian cancer (257). p21$^{Waf1/Cip1}$ inhibits cyclin E/CDK2 complexes and consequently the phosphorylation of p27^{Kip1} that is necessary for its degradation by the ubiquitin pathway. Loss of p21$^{Waf1/Cip1}$ would therefore result in active cyclin E/CDK2 complexes and increased degradation of p27^{Kip1}. Furthermore, bivariate analysis of survival revealed that aberrant expression of p27^{Kip1} did not retain significance when considered with p21$^{Waf1/Cip1}$ expression. Secondly, the multivariate model also included cyclin D1, which was inversely correlated with p27^{Kip1} expression in this study. These data are consistent with results in other tumour subtypes (322), although in contrast to a previous study in ovarian cancer demonstrating concurrent decrease of p27^{Kip1} and cyclin D1 expression in moderately and poorly differentiated tumours (266).

Moreover, p27^{Kip1} expression appears to be induced by increased levels of cyclin D1 in some mammary epithelial cell lines (323). Similarly, levels of cyclin D1 are markedly reduced in embryonic fibroblasts lacking genes encoding for p27^{Kip1} and p21$^{Waf1/Cip1}$, and reintroduction of p27^{Kip1} into these cells appears to restore levels of cyclin D1 (324). The association of cyclin D1 overexpression and reduced levels of p27^{Kip1} in ovarian cancer may represent common independent genetic aberrations that occur in this cancer. As mentioned previously, upregulation of cyclin D1 may be the result of external mitogenic signalling, thereby minimising the effect of p27^{Kip1} regulation of cyclin D1 levels.

5.5   Conclusions and Future Directions

Epithelial ovarian cancer is a heterogeneous disease, constituting several subtypes of tumours that are pathologically distinct and that have subtle biological and clinical differences. Many investigators have reported contradictory results regarding the prognostic significance of abnormal expression of cell cycle regulatory genes in the progression of ovarian cancer, possibly as a consequence of examining epithelial ovarian tumours as a single entity, and generally in a small number of cases. Therefore, careful analysis from large groups of specific subtypes of EOC is required to establish whether the pattern of gene expression co-segregates with clinical and pathological features of malignancy and disease outcome in those epithelial tumours.

Dysregulation of cell cycle genes involved in G_1 to S phase progression is implicated in the pathogenesis of several types of cancers. p53, p21$^{Waf1/Cip1}$ and cyclin D1 have been most extensively studied in ovarian cancer, although the results of associations between aberrant gene expression and clinicopathological features of malignancy and clinical outcome of patients is conflicting. Hence, the aim of this study was to examine the expression of genes along selected pathways, namely cyclin D1/CDK4/p16^{Ink4a} and cyclin E/CDK2/p27^{Kip1}, which are influenced by p53 and p21$^{Waf1/Cip1}$, and that ultimately converge on pRb, in a series of one type of EOC, serous cystadenocarcinomas. The results of aberrant gene expression were correlated to clinicopathological features of serous ovarian cancer and clinical outcome of patients. A further aim was to investigate the various relationships between the protein molecules themselves, in as much as is possible by immunohistochemistry. The major findings of this study are the following:

• This study defines for the first time a significant relationship between aberrant expression of cyclin D1 and clinical outcome in ovarian cancer. Overexpression of cyclin D1 appears to be independently predictive of shorter disease-specific survival in serous epithelial ovarian cancer.

- The results from earlier studies are confirmed regarding the prognostic significance of aberrant p53 and p21$^{\text{Waf1/Cip1}}$ expression in determining clinical outcome in EOC. Although this is the first study to report a significantly shorter progression-free interval in those patients with tumours displaying low p21$^{\text{Waf1/Cip1}}$ expression, this result was not sustained in multivariate analysis when adjusted for other factors. In addition, it was defined that more prognostic information could be gained by simultaneous assessment of p53 and p21$^{\text{Waf1/Cip1}}$ than either molecule alone.

- A differential pattern of p53 expression was reported across serous epithelial ovarian cancers, and tumours displaying moderate expression of p53, i.e. between 5% and 50% appear to have a significantly worse outcome than those with either absent (<5%) or high (>50%) p53 expression. Further p53 mutational and functional studies are required to investigate the significance of this result.

- The study also demonstrates that dysregulation of several cell cycle genes that include p53, p21$^{\text{Waf1/Cip1}}$, p27$^{\text{Kip1}}$ and cyclin E appear to be important in tumour differentiation of serous EOC. Overexpression of p53, loss of p21$^{\text{Waf1/Cip1}}$, reduced levels of p27$^{\text{Kip1}}$ and overexpression of cyclin E all correlated to an increased lack of differentiation of cells.

The possible interactions between the proteins in serous epithelial ovarian cancer were also investigated in this study. Based on the associations between the expression of proteins examined in this current study, a model of cell cycle dysregulation was hypothesised that may account for increased cellular proliferation and consequently poor outcome in serous ovarian cancer. A flow diagram summarising the observations, and hence possible interactions of proteins in G$_1$, is presented in Figure 5.1.

Approximately 60% of tumours in this study demonstrate overexpression of presumably mutated p53 protein, which co-segregates significantly, with reduced

levels of p21$^{Wafl/Cip1}$. Therefore, the mechanism for p21$^{Wafl/Cip1}$ upregulation in serous ovarian cancer, may predominantly rely on transcriptional activation by wild-type p53, which is absent in almost two-thirds of this tumour cohort. Subsequent to the loss of p21$^{Wafl/Cip1}$, the inhibition on cyclin E/CDK2 complexes may be diminished. Active cyclin E/CDK2 complexes phosphorylate p27^{Kip1} that results in increased degradation of this protein by the ubiquitin-proteosome pathway. It was observed that reduced p27^{Kip1} expression in 77% of tumour specimens positively correlated with p21$^{Wafl/Cip1}$ expression but not cyclin E. Therefore, the increased activation of cyclin E/CDK2 complexes, subsequent to reduced p27^{Kip1} and p21$^{Wafl/Cip1}$ levels, may facilitate increased phosphorylation of pRb that results in increased cellular proliferation.

There is evidence to suggest that p21$^{Wafl/Cip1}$ is involved in facilitating the formation of the active cyclin D1/CDK4 complex rather than its inhibition. However, in this study, the level of p21$^{Wafl/Cip1}$ is reduced, although with increased expression of cyclin D1 and reduction of p27^{Kip1} (that is also involved, but to a much lesser degree in inhibiting cyclin D1/CDK4 complexes), the involvement of p21$^{Wafl/Cip1}$ in facilitating the formation of cyclin D1/CDK4 may not be as important. Similarly, with increased levels of cyclin D1, the inhibitory role of p16^{Ink4a} on cyclin D1/CDK4 complexes may also be diminished. Therefore, the net result of this pathway is also likely to be increased phosphorylation of pRb and consequently cellular proliferation.

In summary, dysregulation of one or both pathways that converge on pRb is frequently observed in this study of serous epithelial ovarian cancer, which is likely to result in the rapid proliferation of cells. In addition, the aberrant expression of several cell cycle genes studied, correlates with poor tumour differentiation, and in conjunction with increased cellular proliferation, rapid disease progression and poor clinical outcome in those affected individuals is likely.
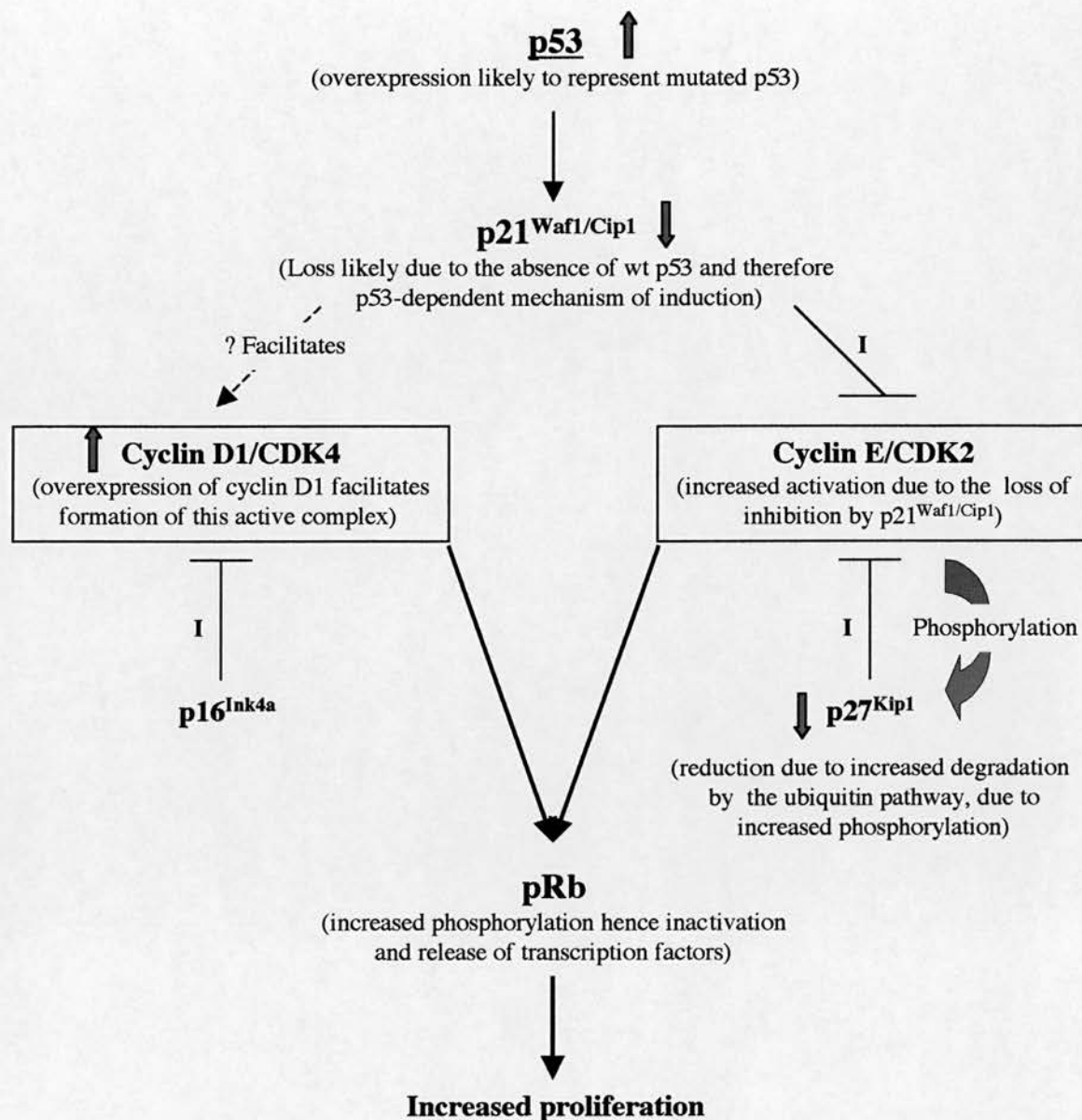
Figure 5.1

p53 mutation resulting in overexpression of mutated p53, leads to reduced levels of p21$^{Waf1/Cip1}$. Consequently, the inhibition on cyclin E/CDK2 is reduced, which results in increased phosphorylation and therefore degradation of p27^{Kip1}, leading to further activation of cyclin E/CDK2. Active cyclin E/CDK2 complexes phosphorylate pRb that subsequently results in increased cellular proliferation. Increased cyclin D1/CDK4 complexes as a result of increased cyclin D1, also phosphorylates pRb and drives cellular proliferation. The inhibitory role of p16^{Ink4a} may be diminished in its effect due to elevated levels of cyclin D1

⬇⬆ observed changes in the expression of genes in our cohort of serous epithelial ovarian cancer

I   Inhibition

Based on the outcome data and the proposed model of the role of these genes in ovarian cancer, the following areas are seen priorities for further research:

- To further evaluate the role of specific *p53* genes mutations that may result in different functional p53 proteins. These proteins may or may not be detected by immunohistochemistry and may have different functional capacities that impact on disease progression in ovarian cancer. Therefore, by correlating p53 immunostaining or simply the presence of gene mutations by sequence analyses with clinical outcome in ovarian cancer, may not be an accurate test for its role as a prognostic indicator of this disease.

- A larger study of serous epithelial ovarian cancers is required to confirm the prognostic role of cyclin D1 in this disease. In addition, the mechanism of cyclin D1 overexpression in ovarian cancer remains to be developed and further investigations of upstream regulators of cyclin D1 is required.

- It is also necessary to evaluate the expression of these cell cycle regulatory genes in large cohorts of other epithelial ovarian cancers that include mucinous, endometrioid and clear cell carcinomas thereby allowing meaningful comparisons of aberrant cell cycle regulation between these tumour types. A major limitation in drawing clinical conclusions from the published literature in ovarian cancer is that the results reported are often contradictory. This may due to incorporation of many types of epithelial ovarian cancers for final analyses in most studies.

In conclusion, this study confirms that dysregulation of cell cycle genes is common in serous ovarian cancers and interactions between protein products are complex. In addition, this study also provides further evidence that the aberrant expression of certain cell cycle regulatory proteins, are independent prognostic markers in this disease.

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