EVALUATION OF NOVEL PROGNOSTIC FACTORS IN OVARIAN CARCINOMA

A thesis submitted to the

College of Graduate Studies and Research

in Partial fulfillment of the Requirements

for the Degree of Doctor of Philosophy

in the Department of Pathology

University of Saskatchewan

By

Kavitha Advikolanu

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SUMMARY OF DISSERTATION

Submitted in partial fulfillment
of the requirements for the

DEGREE OF DOCTOR OF PHILOSOPHY

by

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Abstract

Clinical information was collected from 283 randomly chosen ovarian cancer cases from at the Saskatoon Cancer Centre between the years 1983-1995. The data was evaluated for its significance in predicting survival and relapse free survival (RFS) using univariate and multivariate analysis. Several clinical prognostic factors were identified by univariate analysis. Additionally, using Cox's regression model the independent markers of survival and RFS were FIGO stage, and residual disease in 173 and 178 patients respectively. Data on CA 125 serum level, (available in 89 patients) was a marker of prognostic significance in the patients treated with platinum based chemotherapy. CA 125 and CEA antigen expression were also evaluated in seventy one cases. It was found that mucinous neoplasms exclusively expressed CEA antigen. This study indicates that the evaluation of serum level CEA may be a complementary tool for patients with cancers not expressing CA 125.

In this retrospective study, DNA from paraffin embedded tissue (PET) in patients with ovarian carcinoma was examined to identify gene abnormalities in p53, $p16^{INK4A}$, RB-1, $p21^{WAF1/CIP1}$, Cyclin D1, Erb-B2, and MSH2. Adverse outcome was also examined in addition to survival and RFS, to identify novel molecular prognostic markers. P53 overexpression in 44 of 112 (39%) was associated with reduced survival and RFS ($p=0.04$ and $p=0.008$). Aneuploid DNA content, found in 34 of 112 (30%) cases, was associated with shorter survival and RFS ($p=0.03$ and $p=0.01$). Dot blot hybridization of G1-S control genes ($p16^{INK4A}$, Cyclin D1, RB-1, and CDK4) did not identify amplification or deletion events to be associated with adverse outcome. A number of gene alterations in 59 of 63 (94%) ovarian cancer cases were detected by dot blot hybridization; the lack of

association with clinical outcome indicated that there may be some other genes in addition to those examined that are of prognostic significance.

For eighteen cases, microsatellite instability (MSI) was evaluated by using fluorescently labeled primers at nine loci. LOH was a common event in ovarian carcinoma but MSI was infrequent. Molecular and clinical marker multivariate analysis indicated: a)residual disease for survival, b) stage and residual disease for RFS, were independent markers of prognosis.

Dedicated

To

My Dearest Parents

and

my husband, Rayavarapu Ramakrishna Rao

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Abbreviations

5-mc	5-Methyl cytosine
AJCC	American Joint Committee of Cancer
ALL	Acute lymphoblastic leukemia
APC	Adenomatous polyposis cancer
AS	Advanced stage
ATF	Adenovirus transforming factor-1
ATTC	American type tissue culture
bp	Base pairs
BSO	Bilateral salpingo oophorectomy
CAP	Cyclophosphamide-doxorubicin and cisplatin
CDK	Cyclin dependent kinase
CEA	Carcinoembryonic antigen
CGH	Comparative genomic hybridization
CIP1	CDK-interacting protein 1
CML	Chronic Myelogenous Leukemia
CRC	Colorectal cancer
CT	Computerised Tomography
DCC	Deleted in colorectal cancer
DDI	Delivered dose intensity
dNTPs	Deoxyribose nucleotide triphosphates
EBV	Epstein-Barr virus
ECOG	Eastern Co-operative Oncology Group
EGF	Epidermal growth factor
EOC	Epithelial Ovarian Carcinoma
ES	Early stage
FAP	Familial adenomatous polyposis
FGF	Fibroblast growth factor
FIGO	International Federation of Gynecology and Obstetrics
G.5-Mc	Guanine-5-methyl cytosine
G6PD	Glucose 6 phosphate dehydrogenase
GADD45	Growth arrest and damage factor 45
GOG	Gynecologic Oncologic Group
H&E	Haematoxylin and Eosin
HGF	Hepatocytic growth factor
HGFR	Hepatocyte growth factor receptor
HNPCC	Hereditary Non-Polyposis Colorectal Carcinoma
HPC	Hereditary prostate cancer
HPV	Human papilloma virus
IUAC	International Union Against Cancer
KV	Kilo volt
LFS	Li Fraumeni syndrome
LINE	Long interspersed element
LMP	Low Malignant Potential

LOH	Loss of heterozygosity
LSAB	Labeled streptavidin and biotin
MCC	Mutated in colorectal cancer
MDM-2	Mouse double minute
MDR-1	Multi-drug resistance
MLM1	Melanoma susceptibility gene
MSI	Microsatellite instability
MTS1	Multiple-tissue tumor suppressor gene
MuLV	Murine leukemia virus
N-CAM	Neural crest adhesion molecule
NED	No evidence of disease
NF1	Neurofibromatosis type 1
PBS	Phosphate buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PE	Perkin Elmer
PET	Paraffin embedded tissue
RBF-1	Retinoblastoma binding factor-1
RB1	Retinoblastoma
RDI	Relative dose intensity
REF	Rat embryo fibroblasts
RER	Replication error repair
RFLP	Restriction fragment length polymorphism
RFS	Relapse free survival
RPA	Replicating protein antigen
SDS	Sodium dodecyl sulphate
SSB	Single strand binding protein
SV40	Simian tumor virus
TAH	Total abdominal hysterectomy
TBP	TATA binding protein
TCC	Transitional cell carcinoma
TE	Tris EDTA
TGF-β	Transforming growth factor-β
TGF-α	Transforming growth factor-α
USO	Unilateral salpingo oophorectomy
WHO	World Health Organization

Chapter 1

1.0 Overview

Ovarian cancer is the leading cause of death among all gynecologic malignancies (Silverberg et al., 1990). Epithelial carcinoma is the most common form of ovarian cancer. Nearly 70% of ovarian cancers are diagnosed at an advanced stage (AS) and need aggressive treatments (McNeil, 1995). The overall 5 year survival rates in most countries are generally less than 30% (Beral, 1987). The molecular mechanisms of ovarian cancer development are still widely unknown. Presumably, the behavior is governed by a combination of clinical and pathological factors. The predictive value of selected factors can be estimated utilizing multivariate methods of analysis. Consequently, a mathematical model (prognostic model), may be derived that would be accurate in predicting the length of survival and relapse free survival (RFS) in a specific patient based on known clinical and pathological parameters. Such a model would be valuable for planning future clinical trials, as it would enable patients to be classified into high and low risk groups. The purpose of this study was to evaluate a set of potential clinical and pathological factors and to identify those with independent value for use in constructing prognostic model of disease behavior. The present study represents an analysis of epithelial carcinomas of the ovary occurring in a defined period of time (1983-1995) affecting residents of a defined geographic region (Northern Saskatchewan: north of Davidson) receiving standardized treatment.

Ovarian tumors can be broadly classified into a benign, borderline or malignant category, each with further subtypes. Benign tumors require surgical intervention for

eradication, however they do not recur or metastasize and generally do not decrease a person's expected survival. Benign tumors almost always are serous or mucinous and occur in women between the ages 20 and 60 years. These tumors are usually large, at least 15 cm in dimension. Benign epithelial tumors are typically cystic, hence referred to as *cystadenomas*. Borderline ovarian tumors as recognized by the International Federation of Gynecology and Obstetrics (FIGO) are also known as carcinomas of *low malignant potential* (LMP) according to World Health Organization (WHO) classification (FIGO, 1971, and Serov et al., 1973). In general, borderline tumors are noted in patients older than patients with benign neoplasms and younger than ones with frank malignancies. These tumors are characterized by proliferation of their epithelial cells, especially atypical cell clusters as single or small groups of cells within serous tumors, cellular stratification in mucinous tumors, but an absence of destructive stromal invasion (Ozols et al., 1997). Furthermore, these tumors may also have invasive implants in some cases, and therefore influence prognosis (Bell et al., 1988).

In contrast, the malignant tumors of the ovary, are rare in women under the age of 35 years. The malignant tumors are composed of solid masses of cells, areas of necrosis and hemorrhage as well as infiltrative destructive growth into the stroma. The infiltrative growth pattern is a typical characteristic distinguishing malignant tumors from the borderline tumors with individual, and small clusters of cells observed to infiltrate into the stroma. These tumors relapse, metastasize and decrease survival. Nearly 90% of all malignant tumors originate from the surface epithelium or serosa of the ovary (Dietl and Marzusch, 1993). During embryologic development, the coelomic cavity forms and is lined by a mesothelial lining of mesodermal origin. By a process of invagination, the

mesothelial lining gives rise to the Mullerian ducts, from which arise the fallopian tubes, uterus and vagina. The Mullerian-type differentiations hence share a common background, for example the malignancies arising from: serous resemble the fallopian tube, mucinous resemble the endocervix, endometrioid resemble endometrium and clear cell or glycogen-rich cells resemble endometrial glands in pregnancy. The other histologic types of ovarian tumors that occur less frequently are: 6% sex-cord stromal tumors, 3% germ cell tumors, and about 1`% are unclassifiable tumors (Saigo, 1993).

1.1 Patient factors

1.1.1 Age

The incidence of ovarian cancer increase with age and peaks in the eighth decade, such that the median age of diagnosis 63 (Yancik, 1993). Epithelial ovarian carcinoma (EOC) occurs rarely in women below age 40, after which the rate increases with age from 15 to 16 per 100,000 in 40-44 age group to a peak rate of 57 per 100,000 in the 70-74 age group. Furthermore, the prognosis of younger women is much better than older women with ovarian cancer, probably, due to the higher incidence of borderline tumors and early stage disease at diagnosis (Aure, 1971).

1.1.2 Performance status

The overall measurement of the general condition of the patient is known as "performance status" is indicative of the patient response and survival. The two scales used by clinicians as an indicator of performance are the Eastern Co-operative Oncology Group (ECOG)

and Karnofsky performance scale (Beahrs et al., 1992, Karnofsky and Burchenal, 1949, and Davies, 1986). In ovarian cancer, performance status has been identified as an independent prognostic factor factor in a studies by some investigators (Swenerton et al., 1985, and Klein et al., 1985).

1.1.3 Genetics

Family history compilation is an important information in assigning an individual women's' susceptibility to ovarian cancer. In the general population the lifetime risk is of 1.6% however, a woman with a single family member affected by ovarian cancer has a 4-5% risk (Ozols et al., 1997). Several ovarian cancer susceptibility genes have been identified. It is not known if cancers arising in women with a specific genetic predisposition have different prognoses than those with sporadic cancers (Boyd and Rubin, 1997).

1.1.4 Environmental

Talc, asbestos, ionizing radiation, infections and type of diet are some of the factors that may increase the risk of developing ovarian cancer (Piver et al., 1991). Epidemiologic studies regarding the role of talc (asbestos in talcum powder) and the development of ovarian cancer remains conflicting (Piver et al., 1991, and Hartge et al., 1989). Several case-control studies have detected in the past a statistically significant related risk of 1.4 to 4.8 in women of developing ovarian cancer depending on the type and duration of powder used (Cramer et al., 1982, and Harlow and Weiss, 1989). Some investigators have

indicated the necessity to identify the specific effect of the types of powder used by women since in earlier studies asbestos might have had a confounding effect.

Some studies have shown women in Western countries have an increased risk of developing ovarian cancer possibly due to the consumption of a diet containing high animal fats (Green et al., 1984, and Piver et al., 1991). Other dietary factors such as coffee or tea consumption are not associated with increased risk of ovarian cancer (Green et al., 1984, and Piver et al., 1991).

1.2.0 Treatment factors

1.2.1 Surgery

The treatment strategy for ovarian cancer depends on FIGO stage and age of the patient. In older women, who have completed childbearing, surgical management includes bilateral salpingo oophorectomy, (BSO), total abdominal hysterectomy, (TAH), and surgical staging following which chemotherapy treatment is chosen. However, in young patients with early stage disease, oophorectomy and surgical staging are considered appropriate. In stage IA disease, unilateral salpingo oophorectomy, (USO), and staging is recommended if the contralateral ovary appears to be normal. Grade of the tumor is the most important determinant of prognosis in stage I ovarian cancer cases (Dembo et al., 1990). The 5-year survival rate of grade 1 and stage I patients is reported to be above 95%. On the other hand, the 5-year survival rates for grade 2 or 3 tumors is reported to be between 75% to 80% and 50% to 60%. Other adverse outcome factors in stage I cases include ascites, dense-adherence and clear cell histologic type (Dembo et al., 1990, Monga

et al., 1991, and Young et al., 1990). In addition, the association of capsular rupture with survival in ovarian cancer is conflicting. Although some reports suggest rupture is not an independent prognostic factor (Dembo et al., 1990, Monga et al., 1991, and Sevelda, 1989), others indicate the opposite (Sainz de la Cuesta et al., 1994). Thus surgery alone is adequate treatment for stage IA, grade I tumors (Dembo et al., 1990, Trimbos et al., 1991, and Young et al., 1990).

However, for patients with grade 3 tumors, clear cell histologic type, or possibly dense adherence and ascites, post-operative adjuvant therapy is recommended due to higher risk of recurrence. The necessity of adjuvant treatment for grade 2 tumors is controversial and is compounded by the inter-observer variability in grading. In the high-risk patients adjuvant treatment strategies include single-agent melphalan, intraperitoneal chromic phosphate, and platinum-based combination chemotherapy. In two recent studies some patients treated with melphalan or intraperitoneal chromic phosphate, suffered from either severe toxic reactions or intestinal obstruction (Young et al., 1990, and Soper et al., 1992). Hence, the current adjuvant treatment for stage I high-risk group of patients is platinum-based chemotherapy, which produces excellent survival rates, >90% (Piver et al., 1989, and Dottino et al., 1991). In a recent study at Memorial Sloan-Kettering Cancer Center, there was a significant difference in risk of relapse between patients with grade 3 tumors (51%) compared to 20% for those with grade 1 or 2 tumors (Rubin et al., 1993). Also data gathered on two patient groups in Italy: one patient group treated with cisplatin compared with observation in stage IA and stage IB, grade 2 and 3 and second group treated with cisplatin compared with intraperitoneal chromic phosphate in stage IC patients suggested no significant difference in survival (Pecorelli et al., 1994). Although

platinum-based chemotherapy is recommended for stage I epithelial ovarian carcinoma patients after comprehensive staging, further studies are needed to examine the role of adjuvant chemotherapy in high-risk cases.

In AS disease the primary goal of surgical procedure is to remove a maximum amount of tumor mass (debulking). The standard cytoreductive procedure for these patients consists of TAH, BSO, and omentectomy. Certain novel procedures for debulking include the use of argon beam coagulator, the Cavitron ultrasonic surgical aspirator and various types of laser therapy. The term "optimal residual" disease refers to minimal residual disease, that is no greater than 1cm or 2 cm in diameter following debulking surgery.

In treating advanced ovarian cancer the standard chemotherapy regimen so far has been cisplatin and cyclophosphamide. However, some institutions may prefer carboplatin for cisplatin, based on studies indicating that the carboplatin combination have a superior therapeutic index (Alberts et al., 1992, and Swenerton et al., 1992). However a meta analysis in 1991 of four clinical trials indicated a survival benefit ($p = 0.02$) for cisplatin-cyclophosphamide and doxorubicin (CAP), combination (Cyclophosphamide, 1991) over cyclophosphamide and cisplatin.

Based on recent studies by the Gynecologic Oncology Group (GOG) using the combination of cisplatin and cyclophosphamide compared with cisplatin and paclitaxel, the recommendations for advanced ovarian cancer patients is the latter treatment. The advantage of the latter chemotherapy combination was an increase in the length of progression-free survival in the study. The standard chemotherapy consists of six cycles of chemotherapy at three to four week intervals. With regular checkups during the time,

the patient response to treatment was monitored by physical examination, serum CA 125 levels and imaging techniques such as, computerised tomography, (CT) scan if required. Continuous elevation of CA 125 after three cycles of therapy indicated an unfavorable prognosis. The patients who were disease free are followed up routinely by the Oncologist at regular intervals to monitor relapse. The recurrence rate in these patients is nearly 50% and long-term survival rates (upto 30%) accentuate the fact that progress in this area has been extremely slow.

1.2.2 New Therapy

One of the most promising new chemotherapy regimen has been paclitaxel. Paclitaxel treatment in refractory ovarian cancer patients, phase II trials, yielded response rates from 20% to 37% (Einzig et al., 1994, McGuire et al., 1989, and Thigpen et al., 1994). In addition, based on the data from GOG study showing a potential survival advantage of cisplatin and paclitaxel combination over the cisplatin and cyclophosphamide combination nowadays many oncologists have begun to favor the former as first-line therapy for patients with advanced disease.

Other novel treatment strategies include concomitant chemotherapy and radiotherapy in patients with minimal residual disease, intraperitoneal chemotherapy in patients with minimal disease, and the introduction of dose intensity strategies using either autologous bone marrow rescue or peripheral stem cell support. Furthermore, to ameliorate some of the myelosuppressive effects of chemotherapy, clinicians are also using hematopoietic

growth factors, granulocyte colony-stimulating factor or granulocyte-macrophage colony-stimulating factor.

Topotecan and Navelbine are other new drugs under going investigation for use in treating advanced ovarian cancer patients. Also noteworthy are the use of biologic therapy such as the hormonal agent - gonadotropin releasing hormone analogues, autologous tumor-infiltrating lymphocytes and monoclonal antibodies, and gene therapy.

1.2.3 Residual disease

Residual disease after cytoreductive surgery was found to be related to outcome by several investigators. Surgery as a treatment modality basically contributes to removal of tumor in advanced ovarian cancer without curative intent since the cancer is not just localized to the ovaries. This approach of surgery without expectation of cure is known as cytoreductive surgery or debulking surgery. In 1934, Meigs suggested the concept of cytoreductive surgery followed radiotherapy as adjuvant first-line treatment in ovarian cancer patients would improve survival. However, the benefits of surgical resection were not recognized until the mid 1970's. Griffiths, (1975) reported for the first time improvement in the prognosis of advanced ovarian cancer upon surgical resection of tumors to < 1.5cm residual tumor mass, irrespective of total tumor volume. One explanation is that significant tumor residuum may provide a reservoir of cells that may become chemoresistant when exposed to therapy.

Heintz et al., 1986 suggested surgical debulking procedure in ovarian carcinoma is feasible in 70% to 90% of patients and is associated with 1% morbidity. The rationale for cytoreductive surgery in ovarian carcinoma patients is primarily the physiologic benefit of

removing bulky tumor mass (reviewed in Heintz, 1991). The removal of bulky tumor masses would most likely improve patient's comfort, reduce the adverse metabolic consequences of the tumor, improve her nutritional status, and thereby improve the general condition of the patients. Cytoreductive surgery is also likely to be effective in obtaining adequate drug diffusion and response of the remaining tumor to chemotherapy in solid tumors particularly. The large masses of tumor with a relatively poor blood supply, would create a pharmacologic sanctuary, such that tumor cells are inaccessible to adequate drug concentrations. In addition, such poorly vascularised masses may have low growth fraction (non-proliferating or resting G_0 cells) (Bruchovsky and Goldie, 1982, and Gunduz, 1979). Therefore the increase in the proportion of non-proliferating cells decreases the sensitivity to the cytotoxic effects of drugs. Because G_0 cells are less sensitive to radiotherapy, and also alkylating agents, and DNA binders such as cisplatin, react with or bind to DNA regardless of the cycle phase but kill only those cells that decide to undergo DNA replication, a high growth fraction is crucial for response to therapy. Therefore, cytoreductive surgery may be useful in removing large tumor masses less sensitive to therapy.

In a landmark study, Goldie and Coldman (1979) showed that small tumor masses are not necessarily sensitive in spite of a high growth fraction. Apparently, as the size of the tumor increases, so do the chances of developing drug resistance. Therefore, the possibility of resistant clone of cells emerging to a specific drug is related to number of tumor cells present and the growth rate. Thus, cytoreductive surgery possibly also decreases the chances of developing resistant clones of tumor cells.

In addition, the FIGO classification of 1985, further subdivided stage III patients on the volume of disease at the time of surgery. In a recent study, a comparison of patients with small volume disease, (Stage IIIA and IIIB) and bulky disease (Stage IIIC) suggests that the greater the initial volume of disease the worse the prognosis (Patridge et al., 1992).

1.3.0 Extent of Disease at time of diagnosis

1.3.1 Stage and TNM system

The tumor staging system as devised by the The International Union Against Cancer (IUAC), in cooperation WHO is termed the TNM system (Beahrs et al., 1992, and Hermanek and Sorbin, 1987) with each level in the system associated with a decrease in patient prognosis. In general, this system utilizes three symbols to describe the extent of disease: T = extent of primary of neoplasm; N = condition of regional lymph nodes; M = distant metastases. This system, is extensively used in staging malignant neoplasms of various organs, such as those of the breast, the oral cavity, the urinary bladder, the lung and other organs.

The TNM system is a general method for the clinical staging or description of the extent of the neoplastic disease in the human patient. Tumor stage is defined as the anatomic extent of spread cancer at time of diagnosis.

The staging classification used for ovarian cancer was last revised in 1985 (Staging announcement, FIGO cancer committee, 1986) (Table 1). In ovarian cancer stage IV disease may be diagnosed by cytologically positive pleural fluid or fine-needle aspiration of

supraclavicular lymphadenopathy. In general, tumor staging aids in 1) defining clinical management, 2) facilitating communication between physicians, 3) providing a basis to stratify and analysis of treatment results in prospective studies, and 4) providing prognostic information for patients and their families.

The FIGO classification for ovarian cancer patients has been shown to correlate well with patient outcome. Cumulative series indicate, the 5 year survival rates of patients with EOC are stage I: 50-85%, stage II: 37-79%, Stage III: 7-16%, and stage IV: 2-8% (Swenerton et al., 1985, Klein et al., 1985, FIGO, 1979, Sevelda et al., 1990, and Sigurdsson et al., 1983). In a study by McGowan et al, 1985, the staging assessment at laparotomy by gynecologic oncologist were found to be 97% properly staged, as opposed to 52% and 35% of cases operated on by obstetrics/gynecologists, and general surgeons, respectively. Furthermore, the variations in patient survival and proper staging by a number of investigators underscore the critical importance of staging by an experienced individual for all patients with ovarian cancer. However, besides staging there are other factors that also influence patient therapy and outcome as discussed in other sections.

Table 1: FIGO staging of primary ovarian carcinoma.

FIGO Stage		Tumor characteristics
I		**Growth limited to the ovaries**
	IA	Growth limited to one ovary; no ascites; no tumor on the external surface; capsule intact
	IB	Growth limited to both ovaries; no ascites; no tumor on the external surface; capsule intact
	IC	Tumor either stage IA or IB but with tumor on surface of one or both ovaries; or ruptured; or with ascites present containing malignant cells; or with positive peritoneal washings
II		**Growth involves one or both ovaries with pelvic extension**
	IIA	Extension or metastases to the uterus or tubes
	IIB	Extension to all other pelvic tissues
	IIC	Tumor either stage IIA or IIB, but with tumor on surface of one or both ovaries; or with capsule ruptured; or with ascites present containing malignant cells; or with positive peritoneal washings
III		**Tumor involves one or both ovaries with peritoneal implants outside the pelvis and/or positive retroperitoneal or inguinal nodes. Superficial liver metastasis equals stageIII Tumor is limited to the true pelvis but with histologically proven malignant extension to small bowel or omentum**
	IIIA	Tumor grossly limited to the true pelvis with negative nodes but with histologically confirmed microscopic seeding of abdominal peritoneal surfaces
	IIIB	Tumor of one or both ovaries with histologically confirmed implants of abdominal peritoneal surfaces, none exceeding 2 cm in diameter; nodes are negative
	IIIC	Abdominal implants greater than 2 cm in diameter or positive retroperitoneal or inguinal nodes
IV		**Growth involves one or both ovaries, with distant metastases. If pleural effusion is present, there must be positive cytology to allot a case equal to stage IV. Parenchymal liver metastases equals stage IV**

1.3.2 Serum markers, CA 125, and CEA in ovarian cancer

Antigenic markers are shed into the serum and therefore may be indicative of underlying occult disease and possibly volume of residual disease. Consequently, measurement of serum levels may be useful in monitoring disease progression. In 1982 Weber recognized, the concept of biochemical markers in neoplasms for the first time as being reflective of their phenotype and indicative of basic molecular alterations within the neoplastic cell. A number of neoplasms contain antigens that are both different from (Tumor Specific Antigen) and similar to (Tumor Associated Antigen), their tissue origin, and the clinical utility of antigens produced by some neoplasms has been discerned. The best known examples of antigens in ovarian cancer are CA 125 and carcinoembryonic antigen (CEA). In ovarian cancer CA 125 is a useful guide for determining the disease status of patients undergoing chemotherapy or surveillance following completion of treatment (Onsrud, 1991).

CA 125 is a cell surface glycoprotein of high molecular weight that exists in forms ranging from 220 to greater than 1000 kDA. Nearly 24% of the mass of CA 125 is carbohydrate and its serum half-life is 4.5 days. CA 125 is expressed on the cell surface of amnion, and fetal tissues derived from the coelomic epithelium (O'Brien et al., 1991). In women, CA 125 is found on the surface of epithelial cells of the fallopian tube, endometrium, endocervix, peritoneum, pleura, pericardium, and bronchus (Kabawat et al., 1983a). Occasionally CA 125 may be found in the ovary in inclusion cysts, and benign papillary excrescences of the ovary. The CA 125 antigen is expressed on the surface of ovarian tumor cells with no detection on normal ovarian epithelium (Kabawat et al.,

1983b). The normal physiologic role of CA 125 has not been understood. CA 125 antigen is shed from the cell surface and elevated serum levels are detected in 80-90% of patients with epithelial ovarian cancers. However, CA 125 is not an exclusive marker of ovarian cancer. Elevation of CA 125 in serum have been found in pancreatic cancer (60%), and in some patients with breast, lung, and colon cancers (Niloff et al., 1984a, and Niloff et al., 1984b). In previous studies antibody OC 125 has demonstrated that CA 125 immunolocalization in tumors may provide an indication of the clinical value of serial measurements of CA 125 in patients in whom pre-operative serum levels have not been recorded (Dietel et al., 1986).

CA 125 antigen expression is elevated to greater than 35 u/ml in at least 83% of epithelial ovarian cancer patients (Bast et al., 1983). The potential applications of CA 125 level measurement in ovarian cancer patients have so far included a) follow-up of patients with ovarian cancer, b) pre-operative evaluation of benign versus malignant neoplasms and c) ovarian cancer screening (Gershenson et al., 1996). In the literature it has been reported that CA 125 levels correlate with disease progression or regression in about 80% of cases (Atack et al., 1986, Berek et al., 1986, and Cruickshank et al., 1987). In pre-menopausal women, elevated serum CA 125 levels are non-specific and may be elevated due to non-gynecological carcinomas and benign abdominal disorders (Malkasian et al., 1988). In contrast, however, an elevation of serum CA 125 in post-menopausal women indicates the necessity for prompt surgical exploration as it is more likely associated with ovarian cancer (Ozols et al., 1997).

CEA is a cell surface membrane glycoprotein with a molecular weight of 200,000 kDa (Fuks et al., 1975, and Gold et al., 1978). CEA is expressed in fetal development but is usually not found in adult tissues. CEA serum levels are elevated not only in patients with colon, and pancreatic disorders but also may be elevated in patients with benign gastrointestinal disorders, and in smokers which has diminished its value as a clinical tumor marker. CEA belongs to the large family of closely related cell surface and secreted glycoproteins which represents a subset of the immunoglobulin (Ig) supergene family (Paxton et al., 1987, and Williams and Barclay, 1988). CEA has been shown to function *in vitro* as an intercellular adhesion molecule (Kinugasa et al., 1998, Oikawa et al., 1992, and Benchimol et al., 1989). In one study CEA levels measured pre-operatively, and immunostaining studies were only correlated in 50% of the tumors (Breitenecker et al., 1989). Elevated pre-treatment serum levels of CEA have been detected in 88% patients with mucinous epithelial ovarian cancer (Tholander et al., 1990). Several studies have also shown serum CEA to be useful in differential diagnosis between ovarian, and colorectal adenocarcinomas (Buamah et al., 1990, and Yedema et al., 1992).

In this study CA 125 serum levels were evaluated for their prognostic significance in ovarian cancer patients treated with platinum based regimens.

1.4.0 Tumor specific factors

1.4.1 Histologic types of ovarian tumors

Table 2 represents the current classification of epithelial ovarian tumors, first described by WHO (Serov, 1973). The significance of the classification of common epithelial tumors

lies in apparent difference observed with regard to the biologic behavior, metastatic capacity, the presence of related malignancies, and consequent prognosis including treatment strategy (Dembo and Bush, 1982b, Gershenson et al., 1985, Schray et al., 1983, and Silverberg, 1989). The following section describes the malignant and borderline tumors of the ovary.

Table 2: WHO classification: epithelial ovarian tumors.

Histologic type	Characteristics
Serous	B, LMP, or M
Mucinous	B, LMP, or M
Endometrioid	B, LMP, or M
Clear cell	B, LMP, or M
Transitional cell	B, LMP, or M
Mixed epithelial tumors	B, LMP, or M
Undifferentiated carcinomas	M

B = benign, LMP = low malignant potential, M = malignant.

1.4.1.1 Serous tumors

Serous tumors comprise nearly half of all common epithelial tumors (Russell, 1979a, and Russell, 1979b). These tumors are frequently bilateral (Patricia, 1993) and usually have metastasized at the time of diagnosis (Aure, 1971). These tumors have an overall 5 year survival rate between 20% to 35% (Aure, 1971, and Sorbe, 1982) and hence are considered to be the most aggressive tumors.

1.4.1.2 Mucinous tumors

Mucinous tumors account for the second most common type of ovarian tumors, with only 5% to 20% of them demonstrated a frankly malignant nature (Marsoni, 1990, and Russell, 1979b). Bilateral tumors are noted in 8% to 10% of the cases (Yao and Woodruff, 1994). With about 50% of cases having Stage I disease (Russell, 1979b), the 5 year survival rate for Stage I cases is between 70% to 90% (Aure 1971, and Hart, 1973) but ranges from 40% to 60% for all stages.

1.4.1.3 Endometrioid tumors

Endometrioid tumors are relatively less common tumors of the ovary, accounting for 10%-25% of cases (Czernobilsky, 1970a). These tumors were first described in 1925 by Sampson (Sampson, 1925). Approximately 40% of the cases have bilateral ovarian involvement and nearly 50% patients have Stage I disease (Aure, 1971, and Czernobilsky, 1970a). The microscopic appearance of these tumors closely resembles endometrial adenocarcinomas (Tidy and Mason, 1988) and in about 30% of tumors coexistence of both tumors is noted viz., endometrioid tumor of the ovary as well as that of the endometrium. The prognosis of these tumors is known to be better than serous tumors, with 40% to 60% of patients surviving more than 5 years (Czernobilsky, 1970a, Sevelda, 1990, and Swenerton, 1985).

1.4.1.4 Clear cell tumors

Clear cell tumors account for 5% to 10% of ovarian tumors (Aure, 1971, and Czernobilsky, 1970b) and about 50% of the cases are stage I. These tumors have characteristic clear cells, ("hob nail" cells) and are partially cystic. It is not clear whether the clear cell histologic type comprise the most aggressive kind of ovarian epithelial

tumors. The overall 5 year survival rates were found to range from 50% to 70% by some investigators (Norris, 1971, and Swenerton, 1985). Furthermore, in the past, in stage I the 5 year survival rate was 60% and 12% for all other stages (Jenison et al., 1989).

1.4.1.5 Undifferentiated Carcinomas

Undifferentiated carcinomas lack characteristic histologic features of serous, mucinous tumors other than the defining characteristics of high grade malignancy including rapid proliferation rate. The tumors are reported to account up to 15% of the ovarian malignancies (Hart, 1981, and Marsoni, 1990). These tumors have an overall 5 year survival rate between 11% to 25% (Aure, 1971, Swenerton, 1985, and Silva, 1991).

1.4.1.6 Brenner Tumors

The Brenner tumors, also arising from the surface epithelium, are the least common subtype of ovarian cancer (about 1%) resembling the transitional cells of the urinary tract (Hart, 1981). These are usually benign tumors, although borderline and frankly malignant varieties do occur. Most of these tumors are reported to be stage I cases (Roth, 1985). Minute loci of stromal calcification and ovoid to polyhedral cells with large, longitudinally grooved nuclei (coffee bean-shaped) are typical characteristics of these tumors.

1.4.1.7 Transitional cell carcinomas

A second form of ovarian cancer with urothelial differentiation is the transitional cell carcinoma (TCC) which lacks the benign Brenner component (Austin and Norris, 1987). Patients with transitional cell carcinomas often have AS disease at diagnosis. TCC are considered to be more aggressive than brenner tumors (Austin and Norris, 1987). Despite the spread beyond the ovaries, these tumors are more chemosensitive than the serous and undifferentiated carcinomas (Robey, 1989, Silva, 1990, and Gershenson, 1993).

1.4.1.8 Borderline tumors

Borderline tumors (WHO classification LMP carcinoma) comprise about 4% to 14% of all epithelial ovarian tumors (Aure, 1971, Kliman, 1986, and Russell, 1979a). The median age of patients diagnosed with LMP ovarian cancer is less than EOC cases, and averages between 40 and 54 years (Hopkin et al., 1987, and Kaern et al., 1993). Histologically, LMP tumors are identified on the basis of an absence of destructive stromal invasion, unusual degree of cellular proliferation, epithelial cells appear to be only moderately dysplastic besides maintaining some degree of columnar orientation in most areas, increased mitotic activity and nuclear atypia (Ozols et al., 1997). Out of all the surface epithelial tumors, nearly 32% serous and 50% of all mucinous subtypes occuring are of LMP. Prognosis of LMP tumors is much better than the frankly malignant tumors of the ovary. The 5 year survival rates is usually above 90% in LMP patients with stage I disease (Gershenson, et al., 1996). In a study by Kliman and coauthors,1986, 70% of patients with stage III disease may survive 15 years. The frequency of distribution according to extent (stage) of disease is 73% in stage I, 10% in stage II, 17% in stage III and <1% in stage IV (Reviewed in Chambers, 1989). In the cases with AS disease 20% relapse, despite post-operative treatment, it is in these cases that prognostic markers such as the measurement of DNA content and genomic markers that may be useful.

1.4.2 Grade of ovarian tumors

The grading of neoplasms is primarily based on the degree of dysplasia and refers to the arrangement and size of cells and nucleus. The histologic grading of the tumor is always

done by grading the most dysplastic histologic area of the neoplasm. In general, neoplasms with lower grades have better prognosis than high-grade malignancies, especially when stratified by stage.

The histologic grading of ovarian cancer can be determined by two methods. One method requires the assessment of the pattern of growth of tumor (i.e., papillary structure and glands for low grade versus solid sheets of cells for high grade) (Day et al., 1975). The second method is of grading is based on the cytologic detail (i.e., high nuclear-to-cytoplasmic ratios and nuclear pleomorphism would be considered high-grade tumor) (Decker et al., 1975). Investigators in the past have found grading of ovarian tumors usually has a draw back of inter- and intraobserver variability (Baak et al., 1987, Hernandez et al., 1984, Stalsberg et al., 1988, and Cramer et al., 1987). Grade is closely associated with stage, histologic type and residual disease, and consequently grade has not been found to be an independent prognostic factor.

1.4.3 Molecular markers

1.4.3.1 Clonal Origin of Epithelial Ovarian Cancer

Nearly 90% of tumors of the ovary arise from the single layer of epithelial cells that cover the ovarian surface or that line cysts immediately beneath the ovarian surface. Common epithelial tumors of the ovary (Ozols et al., 1997) is a general term in which tumors of serous, mucinous, endometrioid, clear cell, unclassified, gonadal stromal, and germ cell are included. In 70% of cases ovarian cancer is far advanced at the time of diagnosis with disease disseminated throughout the abdominal cavity. The initial studies undertaken to

understand the origin of multiple lesions in the abdominal cavity by Woodruff and Julian in 1969 led to the belief that many of these deposits were indeed independent primaries. Also, multiple malignancies in the upper genital tract was supposedly believed to be a multifocal disease (Woodruff and Julian, 1969). However, such implications would exclude the possibility of detecting early stage, (ES) ovarian cancer which is confined to the ovaries. The advent of contemporary molecular biology techniques allowed scientists to prove that ovarian cancer is a unifocal clonal disease and not a multifocal disease. To determine the clonality of the disease, three techniques were utilized: measurement of loss of heterozygosity (LOH) at different loci, p53 mutations and X chromosome inactivation. Two independent studies showed 92% (24 of 26) ovarian cancers were of monoclonal origin (Jacobs et al., 1992, and Mok et al., 1992). Indeed, a clonal origin of ovarian cancer favors the involvement of at least more than one mutation in the multistep pathway of tumorigenesis, like colon cancer (Vogelstein and Kinzler, 1993). And presumably each defect in genes contributes to transforming a normal epithelial cell. The inactivation of oncogenes and loss of tumor suppressor genes changes the ability of the cell to grow normally, resulting in uncontrolled proliferation.

1.4.3.2 Types of Mutations

Genes may be inactivated by one of the mechanisms discussed in the following sections.

1.4.3.2.1 Coding region mutation

Coding region of a gene may develop point mutation resulting in inactive or unstable proteins in tumors. Coding region defects are usually due to single nucleotide insertions,

deletions, and point mutations resulting in altered proteins. Tumors occur as a consequence of activating gene mutations in proto-oncogenes, such as ras oncogene in nearly 50% colon carcinomas, and inactivating mutations in tumors suppressor genes (Cho and Vogelstein, 1992). So far, among the abnormalities in expression of tumor suppressor proteins, p53 and RB are well studied examples. The RB gene is inactivated by whole exon deletions or single nucleotide change in bladder carcinoma cells and breast carcinoma (Horowitz, 1989, and Lee, 1987).

Also, transposable elements or insertion sequences such as Alu repeats or insertion sequence elements like retroviral elements can interrupt the coding sequence and inactivate the expression of a gene. An example of this property is Alu insertion into the neurofibromatosis (NF1) gene, resulting in a shift in reading frame and premature termination of translation signal (Wallace et al., 1991). In addition, human LINE-1, (Long interspersed elements) can also generate insertion mutations leading to altered open reading frames (Burwinkel and Kilimann, 1998).

Another key aspect of mutations in coding regions of tumor suppressor genes is the dominant negative fashion with which the mutant allele can disrupt the product of the wild-type allele. The evidence for this view comes from the study of p53 protein.

1.4.3.2.2 Other regions of mutations

1.4.3.2.2.1 Promoter mutations

Promoter sequence mutations in DNA affects the expression of genes and likely contribute to the oncogenic process. It has also been reported the consequence of promoter mutation in RB gene results in loss of transcription from the promoter (Sakai, 1991). Other examples of promoter-region inactivation are unknown in the tumor suppressor

genes discovered so far, probably due to lack of investigation rather than lack of mutations.

1.4.3.2.2.2 Splice site-mutation

The primary RNA transcript (hnRNA) produced in the nucleus usually undergoes RNA splicing to remove intronic sequences (reviewed in Adams, 1996). Depending on the splicing pathway, variation in mRNAs occur resulting in variation in protein species. However, the occurrence of a mutation in a intron-exon boundary could result in spliceosome recognition error in the sequence, and as a consequence a frame-shift followed by premature stop codon in the primary transcript (Soria et al., 1993). These features suggest that aberrant splicing can generate proteins with altered function including frame termination and aberrant cellular localization. This type of splice site mutation has been shown to occur in the p53 gene in hereditary breast ovarian cancer (Jolly et al., 1994).

1.4.3.2.3 Modifier effects

There are a number of mechanisms through which tumor suppressor genes most likely have a modifier effect. The modifier effect is recognized as alteration in the expression of the gene or functions of other gene or its product by hypermethylation, dominant effects, or subcellular localization.

1.4.3.2.3.1 Hypermethylation

Around 3-5% of cytosine residues in DNA are constitutively present as 5-methyl-cytosine (5-mc) (Holliday and Grigg, 1993). Genes with methylated cytosine sequences are apparently transcriptionally active controlling the gene expression pattern (Tilghman, 1993). The variation in cytosine methylation pattern produces DNA with hypomethylation

or hypermethylated regions and might facilitates the carcinogenic process. Hypomethylation occurs in DNA sequences upstream of oncogenes, (reviewed in Counts and Goodman, 1995) and has been detected in human colon cancer. Hypermethylation a second mechanism influencing gene expression inactivates tumor suppressors (deBustros et al., 1988) such as the RB and $P16^{INK4A}$ genes.

1.4.3.2.3.2 Dominant-negative effects

Alfred Knudson (1971) proposed the loss of one wild-type allele results in no phenotypic change because of the compensation by the second remaining allele. The gene therefore acts in a recessive manner, however with a mutation or loss of the second allele, the phenotypic consequence becomes apparent. In such a scenario the dominant-negative regulation by the protein products disrupts tumor suppressor function. One example of the mutant protein blocking the wild-type tumor suppressor activity is that of P53 protein. P53 protein functions normally as a homodimer however in the presence of mutant proteins, it forms an inactivate mutant:wild-type oligomer thus suppressing normal control of cell proliferation (reviewed in Herkowitz et al., 1987).

Recently, cellular oncoproteins have also been implicated in downregulating the function of the tumor suppressor genes. Momand et al., 1992 described the effect of binding cellular protein encoded by the *mdm-2* gene to P53 protein inactivating its function as a transcription factor.

1.4.3.2.3.3 Subcellular localization

The function of proteins is dependent on localization in their appropriate cellular compartments. Thus the dislocation of a protein such as P53 from the nucleus to the cytoplasm is another potential mechanism of inactivation. P53 is a transcription factor and

it is active only at the G1/S phase of the cell cycle. Upon completion of the role at the G1/S phase of the cell cycle, in normal cells P53 protein is inactivated by translocation to the cytoplasmic compartment to permit cell proliferation (Shaulsky et al., 1991). Recent studies have shown that localization of P53 protein is disrupted in not only human breast cancer but also in undifferentiated neuroblastomas, with excessive localization of the protein in the cytoplasm (Moll et al., 1992, and 1995). It seems that the faulty localization also removes the ability of P53 protein to respond to DNA damage thus preventing repair of DNA in the cell before entering G2M of the cell cycle.

1.4.3.2.4 Viral carcinogenesis

Both DNA and RNA viruses have the ability to induce transforming properties in a cell. The oncogenic DNA viruses have genes that encode protein products that bind to specific host proteins (such as the products of tumor suppressor genes) involved in control of cell proliferation (Pitot, 1986).

Viral DNA oncoproteins adenovirus E1A, simian tumor virus (SV40) large T antigen, and human papillomavirus (HPV) E7 complex with RB protein (DeCaprio et al., 1988, Dyson et al,, 1989, and Whyte et al., 1988). The association of the viral oncoproteins effectively neutralizes the negative control of RB on cell proliferation (Whyte et al., 1989, Ewen et al., 1989). The viral oncoproteins also block the function of P53 protein, for example the T antigen of SV40, (Lane and Crawford, 1979) and E6 protein of HPV (Sarnow et al., 1982) by forming protein complexes.

1.4.3.3 General genomic changes

1.4.3.3.1 DNA content

Ploidy is a general genomic marker of instability. There is increasing evidence that measurement of DNA content in solid tumors, such as that of the ovary may be useful in predicting prognosis.

Flow cytometry is a precise method for evaluating the distribution of DNA among the cells of a population (Gray et al., 1990). Typically, cell populations *in situ* are asynchronous and consist of mixtures of individual cells randomly distributed in different phases of the cell cycle. Flow DNA content is based on analysis of single-cell suspension. The cells are stained with a DNA-specific fluorochrome such as propidium-iodide and results are plotted as DNA histograms.

Flow cytometry is considered to be a more objective prognostic variable than grade, which is a marker of biological aggressiveness depending on differentiation of a tumor. In addition, grade also depends on the grading criteria used by the pathologist and suffers from problems including lack of reproducibility.

The term "DNA ploidy" refers to nuclear DNA content as measured by cytometry. In general, solid tumors comprised of cells with a single cycling cell population with a solitary G_0/G_1 peak are classified as diploid (Barlogie et al., 1980). However, tumors which demonstrate an additional G_0/G_1 peak are classified as aneuploid. Because neoplasms are complex mixtures of transformed cells and supportive and reactive stromal with inflammatory cells, there is always a reference "normal" diploid internal control standard for every assay. The presence of aneuploid DNA content or abnormal DNA

content in ovarian carcinomas has been associated with significantly decreased length of survival (Braly, 1992).

Studies so far have been ambiguous about the prognostic value of measuring DNA ploidy in LMP tumors. Some investigators indicate DNA ploidy is of equivocal or no prognostic significance (Anderson, 1991, and Klemi et al., 1988), yet there are other studies suggesting a prognostic role for measuring DNA ploidy (Erhadt et al., 1984; Friedlander et al., 1984, and Kaern et al., 1993). DNA anueploidy is more frequently observed in AS disease compared to stage I and II cases. Furthermore, Kaern et al., 1990, reported a greater frequency of aneuploidy in mucinous tumors (47%) as opposed to serous tumors (15%); however in that series the percentage of patients with AS and mucinous tumors were greater than the serous type. In the series investigated by Kaern et al., 1990, aneuploid cases had a shorter survival period than those with diploid tumors, but the staging distribution offsets some of the significance of the ploidy determined.

1.4.3.3.2 Microsatellite instability (MSI)

The mismatch repair system plays an important role in the maintenance of genetic fidelity during DNA replication, in the outcome of recombinations, and in genomic stability. There are two possible mechanisms producing instabilities in microsatellites. Kunkel et al., 1993 described one such mechanism, ie, DNA polymerase slippage on the repeat sequence during normal replication (Kunkel et al., 1993). According to this model, during DNA replication of the repeated elements on the primer tract, there can be a transient dissociation from the template strand and then realignment in a misaligned configuration (Striesenger, 1966). The process of misalignment of DNA strands can result in mispair of

the molecules such that there is a gain or loss in array of repeated elements. The DNA mismatch repair family of genes would subsequently correct the frameshifts. The Replication error repair (RER+) phenotype is therefore the result of infidelity in the DNA polymerase or the dysfunction of the mismatch repair complex. Therefore, if the unpaired bases are present in the primer strand, continued synthesis yields an elongation of the tract. In contrast, when unpaired bases occur in the template strand, continued synthesis results in deletion.

The second mechanism of instability in microsatellites is due to unequal crossing of DNA between duplexes (Smith, 1973). A normal crossing over of DNA duplexes is one mechanism of homologous recombination during meiosis. Homologous recombination gives genetic variation to future generations by providing genetic material from one parental chromosome and the genetic material from the other parental chromosome by breakage and reunion of the DNA molecules. However, in the absence of normal crossing the chromosomes segregate randomly. It appears unequal crossing over of homologous Alu elements in meiotic cells is the cause of inherited diseases including breast cancer (Puget et al., 1997) and colon cancer (Nystrom-Lahti et al., 1995). Crossing over refers to reciprocal recombination, involving the physical exchange of DNA between homologous chromosomes resulting in a recombinant progeny. Furthermore, crossovers between misaligned repeats results in a deletion from one tract and an addition to another.

DNA based mispairs that remain on the tracts of short repeated sequences are corrected by mismatch repair mechanisms (reviewed by Modrich, 1987, and Grilley et al., 1990). The mismatched repair system has been studied in E. Coli, where well characterized mismatch repair is primarily by the MutHLS proteins. In this system, MutS

binds to the mispair, MutH and Mut L are recruited to form a complex. MutH cuts the unmethylated strand, and exonuclease degradation goes past the point of the mismatch leaving a patch. Single strand building protein (SSB) basically protects the single stranded region across from the missing patch. The repair synthesis and ligation that follow are able to fill the gap (Jiricny, 1994).

In the past, studies in E. Coli DNA polymerase have shown that (GT)n tract errors caused instabilities and that over 90% of them were corrected by mismatch repair systems (Levinson, 1987). In addition, recent studies have shown that the stability of short sequence repeats (poly(CA)), in S. Cerevesiae also depend on integrity of specific mismatch repair systems (Strand et al., 1993). In E. Coli there are nearly 20 known genes involved in DNA replication of repair; also-called mutator genes (Kunkel, 1993). Mutations of mismatch repair genes, such as PMS1, MLH1 and MSH2, result in 100X- to 700X-fold increase of instabilities of (GT)n tracts (Strand et al., 1993). These studies in E.Coli and S.Cerevisiae led to the prediction that instability of the tandem repeats is a consequence of DNA polymerase slippage and a defect in the repair proteins in removing such mismatches.

Screening studies of human colorectal cancers for mutations in simple repeat DNA sequences revealed microsatellite instabilities in mono-, di-, and tri-nucleotide repeats in at least 12-20% of colorectal cancer and about 80-90% of cancers occurring in the "hereditary non-polyposis colorectal cancer" (HNPCC) sydrome (Aaltonen et al., 1993). The Lynch I syndrome describes HNPCC families presenting exclusively with colorectal cancer predisposition (Lynch et al., 1993). This cancer syndrome is distinguished from Lynch II syndrome in which members of families also develop carcinomas of the

endometrium and ovary. The above mentioned studies in E. Coli and S. cerevisiae also led to the proposition that the phenotype of the mutation involved in HNPCC (Aaltonen, et al., 1993, Ionov, et al., 1993, and Peltomaki, et al., 1993 a) is probably due to a defect in a gene required for mismatch repair, such as the human homologue of the mutS-like gene MSH2, or the mutL-like genes, PMS1 and MLH1.

The mechanism of mismatch repair in humans has been delineated as similar to the bacterial system except that there is also a G:T binding protein on the human homologues of mutS, mutL, that is hMSH2, hMLH1, and hPMS2 (Karran, 1995). Genetic linkage analysis of two large HNPCC kindreds detected a locus on chromosome 2, close to a microsatellite polymorphism marker termed D2S123 (Peltomaki et al., 1993a), whereas independent analysis of another family indicated linkage to chromosome 3p (Lindblom et al., 1993). The first HNPCC gene cloned was hMSH2 on chromosome 2p. Gene, MSH2 was discovered by two groups, one group studying a human homolog of the bacterial mismatch repair gene mutS (Fishel et al., 1993). A second group initially localized the HNPCC gene on chromosome 2p to a 0.8-Megabase interval and then showed that the hMSH2 gene is localized within this interval. Further analysis showed that members of families with HNPCC locus mapped to chromosome 2p carried mutations on their MSH2 gene (Leach et al., 1993). An example of the mutation in affected members of one large HNPCC kindred was the C-T transition in one allele that changed highly conserved Proline to Leucine, whereas affected individuals in another large kindred had a C-T transition mutation in one allele that resulted in a nonsense mutation (Leach et al., 1993).

The second gene to be identified was the hMLH1 gene, identified as the human homolog of another bacterial mismatch gene, mutL, on chromosome 3p in several families

(Lindblom et al., 1993, and Nystrom-Lahti, 1994). The hMLH1 gene was found to have a mutation in one of the alleles in affected individuals from these HNPCC families (Bronner et al., 1994). Subsequently, two additional homologs hPMS1 (chromosome 2) and hPMS2 (chromosome 7) were found to be mutated in the germline of a HNPCC patient (Nicolaides et al., 1994). The above studies therefore suggest that HNPCC individuals are heterozygous for these mutations. That is, have a wild-type allele of these relevant genes in addition to the mutated one. While transition from a preneoplastic tissue to tumor tissue can occur through LOH at the Rb locus and mutation in the remaining wild-type allele (Knudson, 1971), in general in the HNPCC individual loss occurs in some patients and, in the case of MLH1, it is the wild-type allele that is lost (Hemminki et al., 1994). Two recent studies demonstrate the consequence of disruption of the DNA repair function and also support the model of accumulation of genetic defects leading to colon cancer (Fearon and Vogelstein, 1990a, Lazar et al., 1994, and Markowitz et al., 1995). Colon cancers that are RER+ tend to develop mutations in genes with polynucleotide repeat tracts in their coding regions including TGFβR2, MSH6, and BAX (Markowitz et al., 1995, and Yamamoto et al., 1998).

Mismatched base pairs in DNA may arise by at least two mechanisms. One of the most common methods is due to replication errors. During replication, the correct base of the mispair is located in the parental strand of the newly synthesized strand and proper correction of the mismatch results primarily in the maintenance of the genomic stability (Modrich, 1991), and secondly, the formation of a heteroduplex between two homologous DNA molecules as part of a recombinational process (Holiday, 1964). Basically if the two DNA strands differ slightly in their sequence due to mutation, mismatches can be formed.

If the mismatch is corrected, all DNA molecules formed will be wild-type homoduplexes. However, in the absence of mismatch correction 50% of the progeny molecules will be mutant homoduplexes (White and Fox, 1974). Furthermore, mismatched base pairs also arise by deamination of 5-mC. The deamination of 5-mC converts a guanine 5-methylcytosine (G.5-mC) bp to a guanine.thymine (G.T) bp (Duncan and Miller, 1980). Correction of this G.T bp to a G.C bp is important to maintain the genetic fidelity. Unlike the deamination of cytidine to uracil which can be repaired by ura-DNA glycosylase, however, the distinguishing feature of the deamination of cytidine is the formation of a normal DNA base thymine, and consequently cannot be repaired by ura-DNA glycosylase. Such cases of mismatched bps can be corrected by the mismatch repair systems in both prokaryotes and eukaryotes (Modrich, 1991). Some investigators have shown that RER+ carcinomas develop exceedingly high rates of microsatellite instabilities (approaching 50% of all microsatellites assayed in the gene).

In the current investigation a subset of ovarian cancer cases were evaluated to assess the role of the RER+ phenotype in ovarian carcinoma using the polymerase chain reaction, (PCR) amplification of nine microsatellites. The nine microsatellites chosen were located on several chromosomes near known cancer genes. MSI markers evaluated in this study were, marker and gene near the marker respectively : D8S254 (NM23-H1), D18S35 (DCC), TP53-Dint (p53), TP53-Pent (p53), D5S346 (APC), D2S123 (MSH2), D1S2883 (HPC1), D3S1611 (MLH1), and D7S501 (MET). Cancers with two or more microsatellite instabilities were considered to be RER+.

1.4.3.4 G1-S control genes investigated in the current investigation

The sequential steps of cell progression through the cell cycle is controlled by genes encoding proteins which in turn create positive{e.g., activated cyclin and cyclin-dependent kinases(cdks)} and negative (e.g., inhibitors of cdk) effects (Sherr, 1993; Hunter, 1993, and Ron, 1994). Dysregulation of these genes can result in premature entry of a cell into the cell cycle without completion of critical macromolecular events (e.g., repair of DNA damage), and subsequently generate genomic instability and neoplastic transformation (Hartwell, 1992). There are at least two stages in the cell cycle that are negatively regulated in response to DNA damage, at G1-S and G2-M check points (Hartwell, 1992, and Pardee, 1989). Consequently, these genes may be the targets of mutation in malignancy.

We have examined the status of the two tumor suppressor genes (RB-1 and $P16^{INK4A}$) along with proto-oncogene Cyclin D1 and CDK4 by dot blot analysis. P53 function is important in regulation of genomic stability and it was also in this project examined by immunohistochemical staining of primary ovarian cancers.

Data on molecular genetic changes in colorectal cancer illustrate a step-wise pathological progression of the disease from normal epithelium into metastatic tumor lesions (Fearon and Vogelstein, 1990a, and Bodmer et al., 1994). The different steps involve either loss or mutation of a number of genes, including the gene APC, K-ras mutation, DCC deletion, and p53 mutation. Mutation in APC tends to occur early in the precancer polyp stage. P53 appears to be a later event. Ovarian cancer is usually detected in AS of the disease and the genetic changes have not been fully understood. In addition a well recognized precursor lesion (like the polyps in colon cancer) has not been detected

making elucidation of sequential events involved in ovarian carcinogenesis difficult. Some think that cancer develops cystadenoma→borderline → malignant, although, this remains to be proven.

The positive regulators of G1-S phase in the cell cycle are a family of protein kinases, the CDK's, that depend upon on the members of the cyclin family for activity (Sherr, 1993, Norbury and Nurse, 1993, and Pines, 1993). The cyclins undergo periodic accumulation and destruction through out the cell cycle such that there is a control of the activities of the various cyclin-kinase complexes during the cell cycle.

The negative regulators are the products of the retinoblastoma gene, RB-1, and the related p107 and p130 proteins (Ewen, 1994). The retinoblastoma gene is a tumor suppresor gene with loss of function through mutations being implicated in both familial and sporadic cancers (Weinberg, 1992a, and Goodrich and Lee, 1993).

In the normal cells, the kinase complexes of Cyclin D with CDK4 or CDK6 promotes progression of a cell in late G1 by phosphorylating RB-1 (Kato et al., 1993). The hyperphosphorylated RB-1 is able to relieve the cell from constraints in G1 phase by releasing the transcription factor E2F required for transcription genes involved in DNA synthesis (Suzuki et al., 1995, and Beijersbergen et al., 1995).

P53 is a tumor suppressor gene that mediates processes such as transcriptional activation of the genes that may in turn regulate cell cycle cycle check point control and apoptosis (Lane, 1992). The wild type p53 regulates expression of p21 (a negative regulator of cell cycle). P53 induces p21 expression upon damage to cell DNA by ionizing radiation blocking entry into S phase which may delay entry to S and allow for DNA repair. Like $p16^{INK4A}$, p21 has the ability to inhibit G1-S progression by inhibiting

the CDK's (Serrano, 1993, and Xiong et al., 1993). P21 has also been shown to block cells in G1 phase by binding and inactivating proliferating cell nuclear antigen (PCNA) (Waga et al., 1994).

The recently discovered $p16^{INK4A}$ gene belongs to a class of CDK inhibitors and behaves as a tumor suppressor gene essential for orderly transition through the G1/S boundary of the cell cycle (Serrano, 1993, and Weinberg, 1995). $P16^{INK4A}$ complexes with Cyclin D1-Cdk4 and CyclinD1-Cdk6 and may regulate CDK4 and D-type Cyclins and mediate phosphorylation of RB1 and RB1-related proteins (reviewed in Sherr and Roberts, 1995). Therefore binding of $p16^{INK4A}$ with CDK4 inhibits CDK4 ability to form complex with Cyclin D and PCNA, preventing phosphorylation of retinoblastoma and progression through the cell cycle.

Therefore the loss of $p16^{INK4A}$ or amplification and overexpression of Cyclin D1/CDk4 would result in the disruption of $p16^{INK4A}$/Rb tumor suppressor pathway. Basically phosphorylation and functional inactivation of pRb leads to unrestrained proliferation. Hence in tumors that have mutation or deletion of the pRb gene, there would be no selection for alterations in the upstream genes. For instance, tumors that overexpress cyclin D1 tend to retain wild-type pRb, whereas cells that are pRb-negative express reduced levels of cyclinD1 (Lukas et al., 1994, and Tam et al., 1994). Also, tumors with mutated pRb express very high levels of wild-type $p16^{INK4A}$, however pRb-positive tumors frequently show loss of $p16^{INK4A}$ or express very low levels (Okamoto et al., 1994, Shapiro et al., 1995, and Washimi et al., 1995).

This study evaluated the role of genes controlling the G1-S phase of the cell cycle in ovarian cancer (Fig. 1). We have conducted a retrospective study in ovarian cancer

patients to evaluate the genomic status of RB-1, Cyclin D1, CDK4 and $p16^{INK4A}$ by dot blot analysis. The significance of p53 nuclear antigen accumulation was also evaluated in the sixty three ovarian cancer cases available for study.

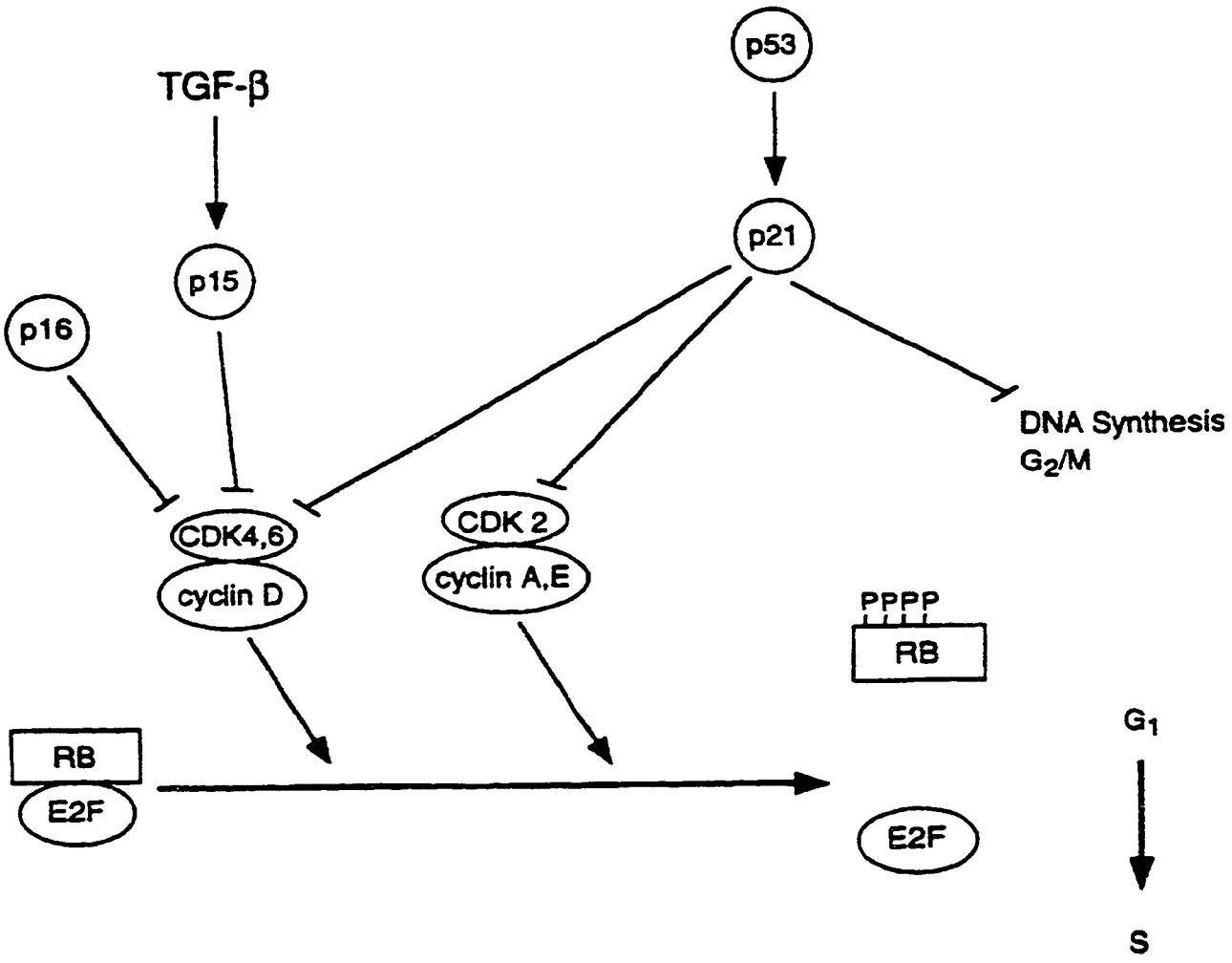

Figure 1: A simplified view of the molecular pathways that control the cell cycle at the G1/S phase and G2/M transition. The Retinoblastoma gene product (RB-1) is the critical cell cycle regulator of transcription factors that are instrumental in the progression of cells through the G1 check point. Inhibition of cyclin/cdk complexes by p15, $p16^{INK4A}$ and p21 prevents phosphorylation of RB-1 and the release of transcription factors necessary for entry into the S-phase. Note: the kinase and phosphatases that regulate cdk's are not shown. (Adapted from Ozols et al., 1997).

1.4.3.5 Alteration in Growth Peptide Factors in Ovarian Cancer

The components of the cell to control proliferation include growth factors, growth factor-receptors, membrane-associated, and cytoplasmic signal transduction molecules, transcription factors (Hollywood and Lemoine, 1992). These can broadly be grouped into proto-oncogene or tumor suppressor gene families. Peptide growth factors are basically products of cellular oncogenes that act by binding to their specific receptors. One peptide growth factor, epidermal growth factor (EGF) and its structural homologue transforming growth factor alpha (TGF-α) stimulate the proliferation of normal ovarian epithelial cells by binding to the EGF receptor (Rodriguez et al., 1991). Several growth factors have been identified, but it should be noted that in human cancer these non-hematopoietic growth factors mediate their effect by receptors containing an intrinsic tyrosine kinase activity (reviewed in Ulrich 1990, and Hunter, 1987). The tyrosine kinase activity is important for mitogenic signalling to the nucleus.

Other protein growth factors like fibroblast growth factor (FGF) and platelet – derived growth factor (PDGF) have also shown to interact with tyrosine kinase receptors (Berchuk, 1990b). Furthermore, transforming growth factor beta (TGF-β) in general is known to inhibit proliferation of normal ovarian epithelial cells (Berchuk et al., 1992a). Previously, the EGF receptor over expression in ~70% of ovarian cancer specimens has been correlated with significantly worse prognosis than in the patients who had lost expression of the EGF receptor (Rodriguez and Berchuk, et al., 1991). Although EGF receptor and its ligand TGF-α are both expressed by normal surface epithelium, it is the persistent expression that is associated with poor prognosis in ovarian carcinoma patients

(Leake and Owens, 1990). However, in some ovarian cancers of epithelial origin TGF-β is not expressed, or unable to be activated and yet in others the inhibitory action of TGF-β is lost (Berchuk et al., 1990a). Hence, in some EOC cases when TGF-β has lost its inhibitory action, such that it mimics cancers from other sites, including breast, lung, colon, and head and neck (Fynan and Schlessinger, 1993).

The c-MET proto-oncogene is the gene near the marker D7S501, encodes a tyrosine kinase cell surface receptor for hepatocyte growth factor receptor (HGFR) (Bottaro et al., 1991). HGFR is a marker of tumor cell motility in carcinomas of breast, lung, head and neck, endometrial, and ovarian.

1.4.3.6 Specific gene and gene product alterations

1.4.3.6.1. Oncogenes in carcinogenesis

Carcinogenesis is a genetically complex process, involving multiple mutations in either one or both of the two families of proliferation control genes, viz., the oncogenes and/or the tumor suppressor genes.

Oncogenes are the mutated counterparts of proto-oncogenes involved in the regulation of cell growth and differentiation. Oncogenes were initially discovered in retro-viruses, but it is now known that viral oncogenes, such as the *src* gene, of Rous sarcoma virus, are actually copies of proto-oncogenes normally present in eukaryotes (proto-oncogenes). The oncogene products so far recognized (protein kinases, cellular signal modulators, growth factors, polypeptide growth factor receptors, and nuclear binding proteins) are important in normal cell differentiation, cell growth and cell division

(Brodeur 1987, and Hunter 1984). The conversion of proto-oncogenes to activated oncogenes as a result of structural changes in the gene itself could be due to point mutation, chromosomal rearrangement, deletion or amplification.

Proto-oncogene activation is therefore a part of a complex multistep process of carcinogenesis in which environmental factors, hormones, and immune reactions are also involved, including products from the various oncogenes being required. Furthermore, some oncogenes initiate malignant transformation while others act as promoters. Also some oncogenes modulate cell growth and capacity for tumor metastasis (modulators).

Therefore, oncogene analysis is becoming a basis of discovering and understanding the origin as well as behavior of tumors. Oncogene studies have proven to be useful in cancer diagnosis and assessing prognosis in a patient with cancer. The tools that are widely used in diagnostic laboratories to detect these changes are DNA/mRNA hybridization studies, and immunonologic detection techniques. The hybridization systems include dot blot, Southern, Northern, and *in situ* hybridization. Immunohistochemical detection systems include Western blot analysis and immunohistochemistry.

Amplification of proto-oncogenes are especially interesting because they have not been noted in benign disorders. Some genes are normally "amplified" i.e. RNA genes. Moreover this mechanism of activation of proto-oncogenes has been correlated with adverse outcome in neoplasms (Masuda et al., 1987). Amplification can be easily detected by dot blot hybridization or by Southern blot hybridization. Amplification can be best assayed by Southern blot hybridization after extracting DNA from tumors and digesting with restriction enzymes. The investigation performed in this project was retrospective

and copy number of target oncogenes/tumor suppressor genes were evaluated in DNA extracted from archival PET.

An example of a relationship between oncogene amplification and prognosis is the presence of *N-myc* amplification in neuroblastoma (Masuda et al., 1987), now considered as an independent marker of rapidly progressive disease in these patients. Amplification of either *ErbB-2*, *int-2* or *c-myc* proto-oncogene has also been shown to imply an unfavorable prognosis in breast cancer (Machotka et al., 1989, and Slamon et al., 1989). ErbB-2 is actually predictive of failure of conventional chemotherapeutic agents and serves as a target for a novel class of therapeutics (Pegram et al., 1998).

Oncogene products detected by immunohistochemical studies have been performed in various human neoplasms, but the results of overexpression should be assessed with extra care, because definitive quantitation is very difficult. In this investigation we have used immunohistochemical studies to determine overexpression of the oncogene product, erbB-2.

1.4.3.6.1.1 ErbB-2

The ErbB-2 (Her-2/neu) proto-oncogene, encodes a 185-kDa transmembrane glycoprotein cell surface receptor. ErbB-2 is a receptor tyrosine-kinase and it normally serves to transmit information across the cell membrane upon stimulation by growth factors. The ligand responsible in activating the tyrosine kinase activity of erbB-2 is a TGFα-like 30 kDa glycoprotein (Lupu et al., 1990). The gene encoding erbB-2 and epidermal growth factor receptor (EGFR) lies on chromosome 17q21-22 and 7p respectively.

The normal p185 is expressed in a subset of neuronal cells and mostly by secretory epithelial cells. In the mesothelial lining erbB-2 is expressed at low to moderate levels (Berchuk et al., 1992c). P185 is normally expressed at low to moderate levels in adult tissue, including kidney, skin, uterus, stomach, lung, colon, ovary (Coussens et al., 1985, and Berchuk et al., 1992b). King et al., (1985) for the first time described the amplification of erbB-2 in a mammary carcinoma.

1.4.3.6.1.2 Cyclin D1

In the past few years there has been remarkable improvement in the understanding of molecules that control transition through the cell cycle. Cyclins and their partners, cyclin-dependent kinases (CDKs), are positive regulators of the cell cycle, that drive cell proliferation forward by phosphorylating specific substrates in a cell-cycle-dependent manner. The CDKs are heterodimeric molecules and are active in the cell cycle progression only on complex formation with cyclins. The D-type cyclins (D1, D2, and D3) form complexes with CDK4 or CDK6 and cyclin E preferentially complexes with CDK2 in the G1 phase (reviewed by Sherr, 1993).

Cyclin D1 is encoded by the CCND1 gene on chromosome 11q13 (Inaba et al, 1992, and Xiong et al., 1992). In 1993, three papers documented the identification of cyclin D1. Motokura et al., (1991) in search of a putative oncogene located on band q11 of chromosome 13 (the site for *bcl-1* gene abnormalities in some lymphomas and leukemias, and amplification events in about 20% of breast cancers) discovered cyclin gene, CCND1 was the same oncogene as PRAD1. Cyclin D1 (PRAD1) is overexpressed in benign tumors of the parathyroid gland because of chromosomal inversion, inv (11)

(p15; q13), whereby CCND1 comes under the control of the parathyroid hormone gene promoter (Motokura et al., 1991). Furthermore, cyclin D1 was also implicated in B cell lymphoma where it is brought under control of the immunoglobulin heavy chain enhancer by a chromosomal translocation at the *bcl-1* breakpoint, t (11; 14) (q13; q32) (Withers et al., 1991, and Seto et al., 1992). These observations implied that cyclin D1 deregulation was causing these lesions and it therefore is an oncogene.

Of all the studies linking deregulation of molecules in cancer, D1-type cyclin has been identified as the oncogene most frequently associated with genetic alteration. Recent studies, suggest that amplification of the 11q13 region is involved in various types of cancers, including breast, and squamous cell carcinomas (Brookes et al., 1992), non-small cell lung, bladder, and esophageal cancer (Jiang et al., 1992).

Also, Cyclin D1 may be activated by retroviral integration, such as in mouse T lymphomas. While T lymphocytes normally do not express Cyclin D1, Friend murine leukemia virus (Mu LV) integration results in transcriptional activation of the gene (Lamme et al., 1992a).

In breast cancer, Cyclin D1 is amplified in 13-20% of the tumors (Lammie and Peters, 1991b, Champene, 1995, and Courjal et al., 1996). In a study by Lamme et al., 1991c, amplification of Cyclin D1 resulted in increased expression of RNA in breast and squamous cell carcinoma cell lines, as well as in primary breast cancers. However, amplification and overexpression was not seen to correlate in all tumors. The absence of DNA amplification in tumors with abnormal levels of Cyclin D1 RNA has been suggested to be due to either undefined mechanisms (Keyomarsi and Pardee, 1993) or as a result of truncation (Lebwohl et al., 1994).

In addition, studies of esophageal carcinomas indicated that tumors that had amplification and overexpression of Cyclin D1 displayed normal expression of Rb gene, however, the subset of tumors that had mutations in the Rb protein did not show amplification and increased expression of cyclin D1 (Jiang et al., 1993). Rb and cyclin D1 are on the same pathway of transformation. If a cell looses either Rb or has upregulation of cyclin D1, transformation will occur. There is no further advantage to loosing the remaining gene.

1.4.3.6.1.3 Cyclin-dependent kinase-4

The cyclin dependent kinase-4 (CDK4) gene is located on chromosome 12q13 (Khatib et al., 1993, and Demetrick et al., 1994) and lies within a region commonly amplified in human sarcomas of various types and in gliomas. The 12q13 amplification in various types of sarcomas ranges between 8% to 36% of cases (Roberts et al., 1989, Oliner et al., 1992; Smith et al., 1992, Khatib et al.,1993, Cardon-Cardo et al., 1994, and Maelandsmo et al., 1995). However, the frequency is nearly 10% in gliomas and anaplastic astrocytomas (He et al., 1994, Rasheed et al., 1994; and Reifenberger et al., 1994). The progression of the cell through the cell cycle is regulated by two key players, one is the regulatory subunit, or cyclin, and a catalytic subunit called cyclin-dependent kinase (Cdk). Thus, the D-type cyclins and their partners CDK4 and CDK6 function as cells leave G_0 and progress through G1. There are two families of specific inhibitors of the Cdks (reviewed in Sherrr and Roberts, 1995). One group, consists of $P21^{CDKN1/CIP1}$, $P27^{KIP1/CK1}$ and $P57^{kip2}$ which function as broad specific inhibitors of cyclin/cdk complexes. $P21^{CDKN1/CIP1}$, appears to be accumulated in terminally differentiated cells and is induced in

response to DNA damage. $P27^{KIP1/CK1}$ levels decline as cells are stimulated to enter the cycle with specific cytokines and accumulates when cells are arrested by TGF-β or contact inhibition. A second family of inhibitors is made of four genes identified so far are, p15, $p16^{INK4A}$, p18 and p19, that specifically inhibit cdk4 and cdk6 and therefore known as specific inhibitors of the cyclin D-dependent kinases (Elledge and Harper, 1994, and Sherr and Roberts, 1995).

1.4.3.6.2 Tumor suppressor genes

Tumor suppressor genes are normal cellular genes which appear to provide a negative signal for cell proliferation. Most tumor suppressor genes behave in an autosomal recessive manner requiring inactivation of both alleles for an effect to be observed (Weinberg, 1992b). LOH is defined as the absence of one of two polymorphic alleles at a locus known originally to be heterozygous, LOH may serve as a marker for loci that encode tumor suppressor genes inactivated by mutation. LOH as a chromosomal mechanism of gene inactivation was shown by Cavenee and associates in the 1980's by the technique of restriction fragment length polymorphism (RFLP) analysis (Cavenee, 1983). LOH presumably is the result of chromosomal lesions such as interstitial deletion, chromosome loss, and duplication or somatic recombination. The limitations of the RFLP technique is that it requires substantial amounts of DNA, and the availability and preparation of a probe at the site of the polymorphism. However, PCR based approaches utilize amplification across polymorphic mini-tandem repeats, are now more convenient. The study of DNA to determine conserved regions of allelic loss is one way to define genetic steps involved in the formation of tumors. Regions targeted or selected for loss

may harbor tumor suppressor gene (s). The tumor suppressor gene, P53 is found to be altered in more than 50% of human malignancies, and Rb gene is involved in retinoblastoma of the eye and other cancers (Hollstein et al., 1991, and Knudson, 1971)

Microsatellite markers, in which the variation is due to differences in length of simple di or tri nucleotide repeats are readily distinguished by use of the PCR. Using specific DNA primers flanking the microsatellite region, PCR amplifies the region containing the di nucleotide repeat, which can then be sized by ethidium bromide stained gel electrophoresis or by detecting fluorescently labeled products on a gel electrophoresis system. The PCR products of these sequence repeats are typically between 100 - 200 bp in length. For example, if in a tumor one repeat allele is missing because of deletion or recombination event, then one of the two allele peaks will be lost. A number of losses of heterozygosity spanning an entire chromosome implies either the loss of the whole chromosome or a somatic recombination event spanning most or all of the chromosome.

1.4.3.6.2.1 Retinoblastoma (RB1)

In 1971 Knudson proposed the double hit model for inactivation of tumor suppressor genes. Retinoblastoma (RB) is a childhood cancer of the eye that occurs as a familial and sporadic cancer. The RB gene was first isolated by three groups in the scientific community (Friend et al., 1986, Lee et al., 1987, and Fung et al., 1987). The RB gene encodes a 4.7 Kb mRNA and 110 KDa nuclear phosphoprotein with ubiquitous expression in normal cells and none or aberrant expression in retinoblastoma cells.

Patients surviving retinoblastoma are also susceptible to other cancers including osteosarcomas, soft tissue sarcomas, leukemia, and lymphoma thereby implicating RB in

non-retinal cancers. Also subsequent mutational studies (reviewed in Goodrich and Lee, 1993) confirmed the involvement of the RB gene in a wide variety of tumors, including osteosarcomas, leukemias, and lymphoma.

The function of the pRB gene product has been found to be disrupted by several tumor viruses including the T antigen of SV40 (De Caprio et al., 1988), the E7 portion of the human papilloma virus (HPV) (Dyson et al., 1989), and the E1A portion of the adenovirus (Whyte et al., 1988). One other mechanism of inactivation of RB gene is through the disruption of gene transcription. In 1991 it was found that CpG dinucleotide regions upstream of the RB gene were hypermethylated in retinoblastomas (Sakai et al., 1991). As a consequence of the presence of methylated alleles there is inhibition of binding transcriptional activators such as adenovirus transforming factor (ATF) and retinoblastoma binding factor (RBF-1).

Furthermore, studies on characterization of the RB gene have revealed its role in cell cycle proliferation. PRb is a nuclear phosphoprotein whose phosphorylation state fluctuates during the cell cycle. PRb is unphosphorylated and active in early G1 phase where it sequesters the E2F family of transcription factors. In the late G1 phase of the cell cycle pRB is phosphorylated and releases E2F, which results in the induction of downstream growth regulating genes such as *C-myc* and *C-myb* and subsequent cell cycle progression. PRb remains dormant until the cell cycle is complete and the kinases responsible for down regulating pRB have been identified as the cyclin-dependent kinases CDK-1, CDK-2, CDK-4 and CDK-6 (Lin et al., 1991, and Lees et al., 1991). The hyperphosphorylated state of pRB is maintained throughout the S, G2 and M phases of the cell cycle. Overexpression of CDK-4 will lead to enhanced progression of the cell

cycle. The cell would thereby be functionally downregulated, proliferating in an uncontrolled manner with oncogenic potential. One of the positive regulators of pRB that has been implicated in the dephosphorylation of this particular protein is protein phosphatase type 1 (PP1) (Durfee et al., 1993, and Alberts et al., 1993). In addition, the mutational inactivation of the phosphatases could lead to constitutive Rb hyperphosphorylation. Clearly, this situation could be potentially oncogenic.

1.4.3.6.2.2 $P16^{INK4A}$

$P16^{INK4A}$ is a 16 Kda protein mapping to chromosome 9p21 identified in 1993 (Serrano et al, 1993) containing four ankyrin repeats. Ankyrin repeats are important for protein-protein interactions and their presence is a hallmark for all members of the INK4 family of proteins. As an inhibitor of cyclin-CDK kinase complex activity, $P16^{INK4A}$ causes G_1 growth arrest.

Initial studies by Kamb et al., 1994a, and Nobori et al., 1994 demonstrated $p16^{INK4A}$ locus to be rearranged, deleted, mutated, or down regulated in a majority of tumor cell lines, including lymphoblastoid lines from affected members of melanoma families. Also, cytogenetic abnormalities of chromosome 9p21 have noted in a wide variety of human tumors, particularly acute lymphoblastic leukemias (ALL) and gliomas (reviewed in Einhorn and Heyman, 1993), indicating that a multiple-tissue tumor suppressor gene (named MTS1) may be present at this locus (Fountain et al., 1992, Petty et al., 1993, and Olopade et al., 1992). In addition, linkage analysis indicated that the region contained a gene involved a melanoma susceptibility gene (MLM1) (Cannon-Albright et al., 1992).

Subsequent work that followed in analyzing the status of this gene in primary tumor samples found the frequency of mutations less than the rate observed in cell lines (Cairns et al., 1994, and Spruck et al., 1994). The reasons to explain these discrepancies include, firstly mutations are an artifact of cell culture and that homozygous deletions of $p16^{INK4A}$ are apparently more common in cell lines than in primary tumors indicating a selection for loss of $p16^{INK4A}$ when cells are forced to grow in vitro (Cairns et al., 1994, Spruck et al., 1994, and Lydiatt et al., 1995). Secondly, there may have been more technical difficulty in obtaining unequivocal results with primary tumor due to contamination of tumor samples with normal cells, thus confounding allele loss studies (Giani and Finocchiaro, 1994, and Lydiatt et al., 1995).

Based on numerous studies to clarify mechanism of $p16^{INK4A}$ involvement in both familial and sporadic cancers, homozygous deletions appears to be the most predominant form of $p16^{INK4A}$ inactivation in various human tumors including gliomas, leukemias, mesotheliomas, sarcomas and ovarian carcinomas (reviewed in Sherre and Roberts, 1995). $P16^{INK4A}$ has also been found to be inactivated by insertions, frameshift mutations, missense mutations, splicing defects (Hussussian et al., 1994, Kamb et al., 1994, Mori et al., 1994, and Liu et al., 1995), chromosomal translocations (Duro et al., 1995) and promoter methylation (Merlo et al., 1994).

$P16^{INK4A}$ mutations are apparently responsible for the disease in at least one third of melanoma families linked to 9p21 (Walker et al., 1995). Nevertheless, there are also studies suggesting deletions rarely being homozygous (Quensel et al., 1995) and 9p21 minimal deleted region of LOH to be outside the $p16^{INK4A}$ locus (Puig et al., 1995).

Thereby the presence of another tumor suppressor gene on chromosome 9p21 responsible for the lower than expected mutations was suggested.

1.4.3.6.2.3 $P21^{WAF1/CIP1}$

When DNA is damaged by ionizing radiation or DNA damaging agents, P53 functions as a transcriptional factor and induces transcription of genes including GADD45 and P21 (WAF1/CIP1/SDI1) (Gujuluva et al., 1994; and Michieli et al., 1994). The induction of the genes GADD45 and $P21^{WAF1/CIP1}$ leads to G1 arrest of cells prior to DNA synthesis or to apoptosis in the cells with wild-type P53. Therefore the $P21^{WAF1/CIP1}$ gene product is a critical downstream mediator of P53-specific pathway of growth in human cells (El-Deiry et al., 1993, and El-Deiry et al., 1994). $P21^{WAF1/CIP1}$ is one of the members of the CDK inhibitors and acts as a tumor suppressor gene in cell lines (Chen et al., 1995). *Invitro* studies have shown that $P21^{WAF1/CIP1}$ can be induced by P53-independent pathways by drugs (Michieli et al., 1994), growth arrest inducing agents (Johnson et al., 1994) as well as by induction of differentiation (Jiang et al., 1994, Steinman et al., 1994, Halvey et al., 1995, and Zhang et al., 1995).

1.4.3.6.2.4 P53

The P53 gene is one of the most commonly altered tumor suppressor genes in human cancer (Caron de Fromentel and Soussi 1992; Hollstein et al., 1991; Tominaga et al., 1992; Levine et al., 1991, and Greenblatt et al., 1994). The P53 gene was first described in 1979 as a protein forming stable complexes with the T antigen of the SV 40 virus (Lane and Crawford 1979, and Linzer and Levine, 1979). The human P53 protein molecule

consists of 393 aminoacids with functional domains, evolutionarily conserved domains, and mutational hot spot regions (reviewed in Greenblatt et al., 1994). The functional domains include the amino transactivation region, the sequence-specific DNA binding region consisting of aminoacids 100-293, the nuclear localization sequence consisting of amino acids 316-325, and oligomerization region made up of amino acids 319-360.

The P53 nuclear phosphoprotein primary effect is to function as a tumor suppressor gene (Lane and Benchimol, 1990). Although initial studies indicated that P53 was found to contribute in the immortalization and transformation of rat embryo fibroblasts (REF) (Eliyahu et al., 1984) and lymphoid cell lines and classified as a proto-oncogene. However, it was later discovered by Hinds et al., 1989, that the original experiments in REF cells utilized the mutant cDNA sequences. Thus, P53 was reclassified as a tumor suppressor gene. P53 gene has been described in detail in section 1.4.3.7.

1.4.3.6.2.5 DCC gene

DCC (deleted in colorectal carcinomas) gene is an important colon cancer gene on chromosome, 18q21, where allelic deletions frequently occur in colon cancer (Fearon et al. 1990b). DCC is also known to be implicated in tumors of the stomach, pancreas, breast, and prostate, and in leukemias (reviewed in Cho and Fearon, 1995). Previously, LOH studies at the 18q locus found, 30% LOH in ovarian cancer cases (Cliby et al., 1993, Osborne and leech, 1994, Sato et al.,1991, and Yang Feng et al., 1993). The DCC gene product was found to have a significant homology to the neural crest adhesion molecule (N-CAM) and the protein functions as a mediator of cell to cell interaction (Fearon and Vogelstein, 1990a). More recent studies on colorectal cancers (CRC) demonstrate that

LOH at 18q has been linked to lymphatic invasion and hepatic metastasis (Jino et al., 1994). In addition, allelic loss of 18q using microsatellite markers were found to be closely related to prognosis of stage II and stage III CRCs (Jen et al., 1994). The most interesting mutation thus far has been the insertional mutation within a TA dinucleotide repeat-rich region within one of the DCC introns (Cho et al., 1994b). The consequences of such splice-site insertional mutation have not been elucidated yet, however it likely would encode truncated (presumably inactive) protein.

1.4.3.6.2.6 APC

The gene near the marker, D5S346, is the adenomatous polyposis coli gene (APC) which is the target gene of familial adenomatous polyposis (FAP). FAP is a relatively rare form of CRC that accounts for 1% of all CRC cases in the age group between 30–40 years (Friedl, 1994). FAP is due to inheritance of a germline mutation of the APC gene. In colon cancers arising in such individuals there is inactivation of the remaining wild type allele (reviewed in Polakis, 1995). Initial studies suggested the involvement of another tumor suppressor gene for the inheritance of FAP, MCC (mutated in colorectal cancer) due to its tight linkage to FAP locus. However, later MCC was discovered to be deleted or mutated in only 15% sporadic colon cancer cases (Solomon et al., 1987, and Kinzler et al., 1991a). The APC gene product is a 300kDa cytoplasmic protein (Kinzler et al., 1991b, and Groden et al., 1991), which functions in the regulation of cell adhesion and cell cycle progression (Peifer, 1996). In a recent study LOH at the 5q locus was detected in 47% (23 of 49) tumours, among these there were 33% (6 of 18) cases with stage I disease

and consequently indicate this may be an early event in the etiology of ovarian cancer (Tavassoli et al., 1996).

1.4.3.6.2.7 TP53-Dinucleotide and pentanucleotide

P53 gene inactivation is a target of frequent mutation in a variety of human cancers (reviewed in section 1.4.3.6.2.4). Analysis of the TP53 dinucleotide and pentanucleotide repeats are useful for identifying LOH at these loci.

1.4.3.6.2.8 MSH2

MSH2 is the human homologue of E. Coli Muts homolog 2 (MSH2). HMSH2 maps to the human chromosome 2p22-21 and linked to microsatellite marker D2S123 (Peltomaki et al., 1993). (Reviewed in section 1.4.3.6.2.8).

1.4.3.6.2.9. HPC1

Hereditary prostate cancer 1 (HPC1) gene locus on chromosome 1q24 -25 is closely linked to a marker D1S2883 (Smith et al., 1996) in a linkage study of families with hereditary prostate cancer (HPC). The existence of HPC1 locus has been reported in the past, however in a recent study there is evidence that the early-onset of prostate cancer was observed in at least 50% of the 91 families (Gronberg, 1997). Role of HPC1 gene in ovarian cancer is unknown.

1.4.3.6.2.10. MLH1

The MLH1 gene is the human homologue of E.Coli MutL mapping to chromosome 3p21-23 and D3S1611 is a second HNPCC locus (Lindblom et al., 1993, and Nystrom-Lahti, 1994). Mutations in MLH1 gene have been reported in 24% of HNPCC families (Han et al., 1995). While LOH is not commonly observed in cancers arising in HNPCC patients unlike many sporadic cancers, in the case of MLH1 and hMSH2 it appears that the wild-type allele is lost (Hemminki et al., 1994). In HNPCC it is hypothesized that in addition to the inherited defect in one mismatch repair gene an additional event occurs to inactivate the remaining gene. Cells with mismatch repair deficiency develop mutations in genes with polynucleotide repeat tracts including TGFβII , MSH6, Bax and others. In one of the studies there were multiple mutations on both APC and TP53 in mismatch-repair-deficient cells (Lazar et al., 1994). Furthermore, it has also been demonstrated that a receptor of growth inhibitor factor, TGFβII, was also target of inactivation and consequent disruption of the receptor protein in mismatch-repair-deficient cells (Markowitz et al., 1995).

1.4.3.7. Human P53

1.4.3.7.1. Biochemical properties of wild type p53

1.4.3.7.1.1. Transcriptional activation

The wild-type p53 protein can act as a transcription factor from the amino terminus. This was demonstrated by fusing the amino portion of the gene with Gal4 DNA-binding protein from yeast. This chimeric Gal4-p53 fusion protein is able to enhance transcription of a

gene with a Gal4 DNA binding element (Fields and Jang, 1990, and Raycroft et al., 1990). P53 mediates transcriptional activation of genes involved in cell cycle arrest upon DNA damage such as the growth arrest and damage factor (GADD45) (Kastan et al., 1992), the CDK-interacting protein 1 (CIP1) (El Deiry et al., 1993), and RB (Osifchin et al., 1994). The protein products of GADD45, CIP1, and RB have been shown to cause growth arrest allowing the cells a chance to repair the damaged DNA. However, if such a repair does not occur successfully, the presence of normal P53 may alternately induce programmed cell death (apoptosis) (Lane,1993, and Hoffman and Liebermann, 1994). P53 transactivates the bax gene (Selvakumaran et al., 1994), a modulator of apoptosis, and the mouse double-minute, (mdm-2) gene (Barak et al., 1993) most likely for the initiation of negative feedback control, as well as the thrombospondin gene (Dameron et al., 1994) considered to be an inhibitor of angiogenesis.

1.4.3.7.1.2. Transcriptional repression

P53 binds to genes that are transcription regulating proteins such as TATA binding protein (TBP) and inhibits its function as a basal transcription factor (Seto et al., 1992). Apparently the genes repressed by p53 lack p53-responsive elements (Mack et al., 1993) and are dependent on p53 oligomerization state for their transcription. An example of such repression is the proliferating cell nuclear antigen (PCNA), that activates DNA polymerase δ, one of the components of the DNA replication machinery (Subler et al., 1992). This is the result of direct interaction between p21 and PCNA as downstream effectors of p53 (Waga et al., 1994) thereby allowing DNA repair.

1.4.3.7.1.3. P53 protein-protein interactions

P53, in addition to inducing and repressing gene expression, also inhibits DNA synthesis by a transcription-independent mechanism that functions through p53 protein-protein complexes, involves the replicating protein antigen (RPA) (Dutta et al., 1993). Furthermore, p53 also forms complexes with excision repair factor ERCC3 (Wang et al., 1994).

1.4.3.7.2. Biological Functions of p53 on DNA damage

The biochemical properties described above correlate with the biological function of this molecule as described in this section. Basically there are two distinct pathways that p53 can take to maintain genomic integrity. In response to DNA damage, p53 is induced and either stops cell proliferation (by arresting the cell cycle) allowing the repair of damaged DNA, or directs the cell to apoptosis (Lane, 1992). However, tumor cells lacking intact p53 fail to induce G1 arrest and may careen on a genetically less stable pathway toward mutations and chromosomal rearrangements, eventually leading to rapid proliferation of malignant clones (Lane, 1992, Vogelstein and Kinzler, 1992, Marx, 1993, and Kuerbitz et al., 1992).

P53 mediated cell cycle arrest genes identified so far include GADD45, MDM2, Bax and $p21^{WAF1/CIP1}$. Although GADD45 is the first gene induced by ionizing radiation in a p53-dependent pathway (Kastan et al., 1992) and is found to be associated with both inhibition of cell growth in vitro and DNA repair (Smith et al., 1994), its function(s), in cells is still elusive. Bax is a protein induced after DNA damage that promotes cell death (Miyashita et al., 1994, and Kitada et al., 1996). MDM2 is a cellular oncogene whose

product can inhibit p53 protein function by controlling cell cycle progression and/or cell viability (Momand et al., 1992, Wu, 1993, Chen, 1994, and Jones, 1995).

It was also shown by overexpression of wild-type p53 in various carcinoma cell lines that p53 mediates cell cycle arrest (Baker et al., 1990, and Diller et al., 1990). Though p53 induces a number of genes that contain p53-repressive sites upon DNA damage such as GADD45, MDM2, Bax and p21 $^{WAFI/CIPI}$, but p53-mediated cell cycle arrest is associated directly through induction of p21 $^{WAFI/CIPI}$ (El-Deiry et al., 1993). P21$^{WAFI/CIPI}$ protein complexes with cyclin/CDK, thus inhibiting CDK activity (Harper, 1993, and Xiong, 1993). Therefore, p53 causes a G1 arrest by maintaining pRb in its active state and preventing the action of transcription factor, E2F, required for G1-S transition.

The role of p53 in the regulation of DNA damage-induced apoptosis was first suggested by the studies with the myeloid leukemia cell line M1, whereby induction of p53 expression was correlated with apoptosis (Yonish Rouach et al., 1991). The pathway of p53-dependent apoptosis was further corroborated by the lack of apoptosis in p53 null mice subject to irradiation (Merrit et al., 1994), and in cells derived from such mice after growth factor deprivation (Lotem and Sachs, 1993), as well as by loss of apoptotic function in cells with mutant p53 (Zhu et al., 1994). Furthermore, p53 induces apoptosis by down-regulating the bcl-2 gene and up-regulating its partner, the cell-death promoting factor, bax (Selvakumaran et al., 1994, and Miyashita et al., 1994).

Since there are two distinct pathways that p53 can take upon induction by DNA damage, several experiments have been carried out in the literature to understand the "decision fork" of arrest or apoptosis. It has been suggested that certain types of cells in

the case of tumors from the breast, lung, colon, pancreas primarily use p53 for G1 arrest (Slichenmeyer et al., 1993). However tumors of cells from lymphocytes responding to DNA damage use p53 induction to mediate apoptosis (Slichenmeyer et al., 1993). Moreover, the "decision fork" of arrest versus cell death was further investigated and found to depend on the presence of a growth factor in a murine hematopoietic cell line (Canman et al., 1995). The investigation using murine hematopoietic cell line suggest that the growth factor provides a signal for growth arrest/survival and removal of growth factor enhances cell death. Consequently, it has been suggested that the anti-erbB-2 antibodies may be working through this type of mechanism in human breast and ovarian carcinoma cells (Pietras et al., 1994). Hence inhibition of the "survival signal" initiated by growth factors is one potential clinical application of understanding p53 function in cell death. There are a number of other potential targets being considered within these p53 signalling pathways to enhance therapeutic outcome (reviewed in Harris, 1996).

1.4.3.7.3 Loss of heterozygosity on 17p and P53 mutation

One of the mechanisms of P53 as inactive is frequently through LOH at the p53 locus and mutation in the remaining allele (Knudson 1971, Levine and Momand 1990, and Weinberg, 1992). Introduction of wild-type P53 into malignant cells (Baker et al., 1990, Diller et al., 1990, and Mercer et al., 1991) results in cell cycle arrest.

In some tumors, such as that of colorectal carcinomas the region of 17p within bands 17p12 to 17p13 where the p53 gene is localized is frequently deleted (Kern et al., 1989, and Baker et al., 1989). In addition, studies in medulloblastoma and malignant astrocytoma also showed that the p53 locus was frequently a region of deletion (Cogen et

al., 1990, and Fults et al., 1992). These initial studies and subsequent work (Khine et al., 1994, Kohler et al., 1993, and Jego et al., 1993) demonstrated that loss of p53 allele reduces the concentration of wild-type p53. Moreover, one allele of p53 develops a missense mutation and the other allele is subsequently lost by deletion of chromosome 17 (usually through chromosome loss as a result of non-dysjunction or mitotic recombination) thus removing all residual wild-type p53 from the cell as well as removal of p53 growth control. Tumors of the breast and liver, though displaying evidence of p53 mutation and 17pLOH, frequency of the allelic loss on 17p is apparently higher than the occurrence of p53 mutations in these cancers (Hollstein et al., 1997). Furthermore, a second suppressor gene on 17p involved in breast cancer (Coles et al., 1990), and in hepatocellular carcinogenesis has been inferred (Fujimori et al., 1991).

1.4.3.7.4 Immunohistochemical analysis of p53 protein

The p53, protein in addition to providing a mechanism of malignant transformation, has been studied as a tool for cancer prognosis. In 1982, Benchimol and coworkers developed a radioimmuno assay to show the overexpression of p53 protein in transformed cells and undetectable in normal cells by this assay. In the numerous studies that followed these results were confirmed and typically p53 protein accumulation in neoplasia is primarily due to missense mutation(s), resulting in amino acid substitution that modifies the conformation and stability of the protein. The conformational change prolongs the half-life of the p53 protein from 6 to 20 minutes for normal cells up to 6 hours, thereby allowing its accumulation in the cell nucleus (Maltzman and Czyzyk, 1984, and Kastan et

al., 1991). There is also a transient rise in the level of p53 in response to DNA damage by radiation (Kastan et al., 1992, and Lu and Lane 1993).

A majority of human malignancies tested so far have been shown to overexpress the p53 protein (Bartek, 1991, Gusterson, 1991, Wynford-Thomas, 1992, and Hall, 1994) with the p53 antibody. The simplicity and rapidity of the assay has led to analysis of thousands of cases. Furthermore, there is a good correlation between the frequency of p53 overexpression by immunohistochemistry and the frequency of mutations detected directly by DNA sequencing tumors of lung, colon and ovary (Greenblatt, 1994, and Hollstein et al., 1997). Mutant p53 proteins stabilized due to missense mutation are generally concordant in the two assays. However, for tumors with frameshift and nonsense mutations that result in no protein product or production of a truncated and unstable protein (account for less than 15% of sequenced mutations and are expected to inactivate only one allele) are usually immuno-negative, including mutation in RNA splice site for some tumors (Tominaga, 1992), such as melanomas reducing the sensitivity of immunohistochemical assay.

There are a number of monoclonal antibodies to p53 protein that can be used effectively on either fresh or fixed tissues. Some of these commercially available antibodies are CM-1, D01 and D07 (Iggo et al., 1990, Midgley et al., 1992, Baas et al., 1994, and Soussi et al., 1994).

1.4.3.7.5 P53 mutations in sporadic cancer

In the recent years several consistent characteristics of p53 gene mutations have been found through analysis of a huge number of human cancers (Hollstein et al., 1991, and Chang, 1993). These studies suggest that, depending on the site and nature of DNA changes in particular tumors, it may be useful in identifying factors such as occupational exposure to carcinogens, specific dietary practices, or exposure to cigarette smoke (Chang, 1994).

The most frequent p53 mutations common to most cancers is a point mutation within the coding sequences of the p53 gene, giving rise to single base substitution rather than an altered protein (Hollstein et al., 1991, Chang, 1993, and Soussi, 1994); with the exception of melanoma and cervical carcinoma. It seems in carcinomas of the uterine cervix, the HPV-encoded (E6) oncoprotein results in functional inactivation of wild-type p53 protein through binding and ubiquitin dependent degradation (Crook, 1991, Scheffner, 1991, Iwasaka, 1993, and Kurvinen, 1994). The second p53 point mutation, particularly common in cancer of the colon, brain and in hematological malignancies, is the high frequency of C to T transition at CpG dinucleotides (Hollstein et al., 1991, Jones et al., 1991, and Greenblatt et al., 1994). Apparently, the CpG sites are hotspots for G:C to A:T (Coulondre et al., 1983, and Cooper and Krawczak, 1990) due to spontaneous deamination of 5-mc residues found at CpG dinucleotides in mammalian genome and/or by enzymatic deamination of cytosine by DNA (cytosine-5)- methyl transferase when S-adenosylmethionine is in limiting concentration (Wink et al., 1991, and Shen et al., 1992).

Thirdly, a characteristic of missense point mutations found in human malignancies is the tendency to fall at codons corresponding to amino acids that are evolutionarily conserved. There are 23 CpG dinucleotides in the mid-region of the p53 gene and primarily all tumor CpG transition mutations are noted at one of six sites (codons 175, 196, 231, 248, 273, and 282) (Greenblatt et al., 1994). The common feature of these hotspots is their presence in either an evolutionarily conserved domain and at a conserved amino acid, or CGA triplets at which a C to T transition in the first position of the codon would lead to a chain termination signal and loss of tumor suppressor function. Finally, a typical pattern regarding p53 mutations in human cancers has emerged. There are basically seven major categories of point mutations (six major base substitution classes, and base addition/deletions) (reviewed in Hollstein et al., 1997). Primarily at CpG dinucleotides, a G to A substitution in colorectal cancer, and G to T transversions in lung cancers are the most frequent type of substitution detected.

1.4.3.7.6 Molecular pathways of p53 inactivation

1.4.3.7.6.1 Germline mutation

Germ line p53 mutation is a rare cancer syndrome which was first described by the existence of both a proband with a sarcoma and two other first-degree relatives with a cancer (below age 45) (Li et al., 1988). Germline p53 gene mutations have been associated with the early-onset of cancer known as Li-Fraumeni Syndrome (LFS) families (Malkin et al., 1990). The affected family members with LFS syndrome carry one mutant and one wild-type p53 allele and have a 50% incidence of cancer by age 30. Neoplasms that arise in these families are mostly breast cancer, soft tissue sarcomas, osteosarcomas,

brain tumors and leukemias and, less commonly, adrenocortical carcinomas, melanomas, gonadal germ-cell tumors, and carcinomas of the lung, pancreas, and prostate (Frebourg et al., 1992).

In addition, although the many germ-line mutations detected in LFS are similar to somatic mutations in tumors such that transcriptional and tumor suppressor function of the wild-type p53 protein is suppressed. However, recent studies by Birch et al., in 1994 suggest the presence of mutation outside the p53 protein encoding region. There were no mutations detected in the coding sequence in a family despite the overexpression of p53 protein as detected by immunohistochemistry (Birch et al., 1994).

1.4.3.7.6.2 Somatic mutations

Mutations of the p53 gene take place in more than one half of human cancers (Harris, 1991, Hollstein et al., 1991, Chang, 1993, and Soussi et al., 1994). Missense mutations comprise nearly 90% of the substitution mutations or hotspot mutations that occur in the evolutionarily conserved regions between exons 5 and 8. Since the mutant p53 protein is resistant to cellular degradation, mutant protein accumulates within the nucleus leading to overexpression, which can then be easily detected immunohistochemically. Therefore, immunostaining is useful as a rapid technique to screen for cancers with probable p53 mutations. Furthermore, recent crystallographic studies have shown that the hotspots mutations in tumors come in close contact with the p53-DNA interface (Cho et al., 1994a, and Prives, 1994). This p53-DNA interface is key to tumor suppressor function and is disrupted due to wrong amino acid substitution because of a missense mutation. The mutational hotspots located at the amino acid residues 175, 248, 273 and 282 have been

identified in a variety of cancers (Hollstein et al., 1991, and Chang, 1993) and account for nearly 30% of all p53 mutations. Tumors with nonsense, frameshift or splice site mutation account for almost 5% of lung, esophageal carcinomas (Hollstein et al., 1991).

1.4.3.7.6.3 Viral oncoprotein and p53 protein complexes

P53 protein function can be disrupted by binding to a viral protein such as SV40 T antigen with which it was originally identified. P53 protein-virus mediated oncogenesis include the HPVE6 protein (Werness et al., 1990) involving cervical cancer, the X protein of hepatitis B virus (Wang et al., 1994) involving hepato-cellular carcinomas and Epstein-Barr Virus (EBV) EBNA-5 (Szekely et al., 1993) encountered in EBV-associated nasopharyngeal carcinomas and lymphomas (reviewed in Chang et al., 1995).

1.4.3.7.6.4 Binding to cellular gene products

P53 can also be down regulated through association with cellular proteins encoded by an oncogene mdm-2 (Fakharzadeh et al., 1991) which is believed to be amplified and overexpressed in nearly 30-60% of cancer (Cordon-Cardo et al., 1994). While, mdm-2 amplification and p53 mutations are seen in 10% of human sarcomas and this group has a relatively poor long-term survival than sarcoma patients with no p53 or mdm-2 gene mutations or the group with p53 or mdm-2 mutations alone (Cordon-Cardo et al., 1994).

1.4.3.7.6.4 Oligomerization of p53 oncoprotein

It is believed that p53 protein exists in cells as oligomeric complex (Kraiss et al., 1988). The interaction of mutant p53 with wild-type p53 results in mixed oligomers and drives a

change in conformation of the wild-type to the mutant form (Milner and Medcalf, 1991). The mutant p53 can act in a dominant-negative way, whereby such mutants bind and inactivate the products from the wild-type p53 gene (Finlay et al., 1988). Thus, explaining the loss of p53 function which can explain that p53 contributes to cell transformation and acts as an oncogene (Finlay et al., 1989). Recently, in reference to the dominant-negative hypothesis, it has been demonstrated that in the presence of mutant p53, wild-type p53 can no longer bind DNA and function as a transcription factor (Kern et al., 1992, Unger et al., 1992, and Srivastava et al., 1993). More recent work in transgenic mice, found the expression of dominant-negative mutants of p53 only enhanced malignant transformation in the presence of wild-type p53 in stark contrast to animals nullizygous for p53 (Harvey et al., 1995). Brachmann et al., 1996 have further indicated that dominant-negative p53 mutations probably contribute to a greater proportion of human cancers than recessive ones.

1.4.3.7.6.5 Alteration of p53 subcellular localization

P53 protein may also be inactivated by dislocation from the nucleus to the cell cytoplasm where it is known to be nonfunctional (Shaulsky et al., 1991). P53 is a transcription factor and the p53 protein when present in the nucleus can act as a negative regulator of cell proliferation. The transcription regulatory function is lost when the p53 protein is sequestered into the cell cytoplasm; transcription which is absolutely critical for its role as a tumor suppressor (Pietenpol et al., 1994, and Crook et al., 1994). Moll et al., in 1992 showed that the wild-type p53 in some human breast cancers is localized in the cell cytoplasm.

1.5.0 Metastasis

It is thought for the tumor to metastasize a subset of tumor cells should have angiogenic activity (Folkman, 1993).

1.5.1 NM23-H1

NM23-H1 is a tumor suppressor gene that encodes a nucleoside diphosphate kinase. NM23-H1 confers a metastatic property to cells and its expression is reduced in highly metastatic cells. The molecular properties of NM23-H1 and its role in the progression of cancer have been reviewed by de la Rosa and colleagues (1995).

1.6.0 Other tumor markers

1.6.1 CA125/ CEA by immunohistochemistry

Immunohistochemical staining of CA 125 has been demonstrated on fresh tissue as well as from formalin-fixed tissue (Kabawat et al., 1983a). The CA 125 gene or complementary DNA sequence have not been found so far. CA 125 carries two antigenic domains: one is the domain binding monoclonal antibody OC 125 and second is the monoclonal antibody M11 binding domain. M11 antibody recognizes a high-molecular-weight subspecies similar to OC 125 in the glycoprotein that expresses CA 125 antigen (O'Brien et al., 1991). The advantages of using the M11 antibody is its ability to detect the CA 125 antigen in formalin-fixed and paraffin embedded tissue (PET) sections. Most likely the epitopes detected by M11 are less subject to loss when the tissue is formalin-fixed and

paraffin embedded, compared with the epitopes recognized by OC 125. Since this was a retrospective study we have evaluated CA 125 antigen in the PET by using M11 antibody (O'Brien et al., 1991).

In the past Charpin et al., determined that CEA immunohistochemical detection may be useful in monitoring ovarian cancer (Charpin et al., 1982). Tissue expression of CEA is observed in 15% of benign, 80% of borderline, and 100% malignant mucinous neoplasms of the ovary (Charpin et al., 1982). In the same study, CEA positivity was also noted in 30% of endometrioid carcinomas, 50% of malignant mixed mesodermal tumors, 14% of clear cell carcinomas, and 36% of brenner tumors (Charpin et al., 1982). CEA expression has also been demonstrated in occasional teratomas and Leydig cell tumors; and in metastatic carcinomas from the breast or gastrointestinal tract (Fenoglio et al., 1981).

In this retrospective investigation of ovarian cancer we have evaluated both CA125 and CEA immunoreactivity in PET for their value as prognostic tumor markers.

Chapter 2

2.0 Rationale of the present investigation

The rationale of the present investigation is to identify clinical and molecular factors related to survival and RFS in ovarian cancer. Ovarian cancer has spread beyond the ovaries in at least three quarters of women at time of diagnosis. A spectrum of ovarian epithelial neoplasia exists, ranging from clearly benign to those of intermediate or LMP ("borderline") to the invasive malignant tumors. The most important predictors of survival appears to be FIGO stage at time of diagnosis. Patient survival is closely related with FIGO stage at diagnosis, ranging from 80% to 90% 5 year survival rate for stage I tumors and to less than 5% 5 year survival rate for stage IV disease. Although, stage is an important predictor of survival, it has many problems and lacks precision, particularly in patients with stage II and III disease. The overall survival of ovarian carcinoma patients is 25 to 30% at 5 years (Beral, 1987). Primary tumors of the ovary may arise from surface coelomic epithelium, germ cells, or stromal elements of the ovary. Benign ovarian tumors tend to occur in younger women, while malignant tumors are more frequent in the older women. Among the cancers of women, ovarian cancer constitutes 4% of malignancies in women and accounts for 5% of cancer related deaths in these women.

The underlying molecular alterations in ovarian cancer are essentially unknown. Cancer appears to be a multistep process involving mutations of genes that are implicated in genomic stability, proliferative rate, apoptosis, invasiveness, metastasis and drug resistance. In colon cancer a pathway of multiple genetic alterations linking benign adenomas to invasive cancer has been described by Vogelstein and coworkers (1993).

Colon cancer is one of the best understood human solid cancer and serves as a model for understanding the process of solid tumor oncogenesis at other sites.

Providing optimized individualized treatment, and monitoring its effects in ovarian cancer patients is a challenge to both the clinical oncologist and pathologist. In EOC treatment decisions are made on the basis of histologic subtype and FIGO staging system alone. One problem is that this staging system has subjective components (Dembo 1982a). There are a number of clinical parameters that have been useful in estimating a patient's prognosis. Age, FIGO stage, ascites, malignant histologic type, residual disease, grade, and performance status are proven to have value as clinical prognostic markers. Unfortunately many of these conventional markers used to predict tumor behavior are subjective, semiquantitative, and interobserver reproducibility is poor (Baak et al., 1987). The development of ultimate prognostic model likely depends on stage at the time of diagnosis, aggressiveness, response to treatment and the patient constitution (Boente et al., 1994). In 1993 the AJCC had proposed a criteria for evaluating any putative prognostic factor, that is these factors must be a) significant: meaning the prognostic factor occurs rarely by chance b) independent: referring to the factor retaining its prognostic value when new prognostic factors are added and c) clinically relevant, that is it affects patient management and decisions. The TNM staging system in the 1950s for cancer and FIGO staging system for Gynecologic cancers in the late 1970s and early 1980s emerged as criteria for a universal staging system (Hermanek and Sorbin, 1987, and Beahr et al., 1992). These staging systems have been used for making treatment decisions, selecting patients for clinical trials, analyzing data from clinical trial and communicating prognosis to patients. (Burke and Henson, 1993). It was also highlighted

that the goals of any useful prognostic system include accuracy and usefulness. Consequently the accuracy of a prognostic system or will be dependent on the accuracy of the components used in its construction. Systems that are less dependent on subjective criteria will likely be more accurate and have greater reproducibility. If such a functional or "worthy" model can be obtained, it will be very useful for constructing clinical trials, analyzing outcome data, and ultimately for stratifying patients into separate treatment groups to optimize therapy and improve outcome.

Emerging diagnostic techniques, molecular tests (William, 1991) and novel treatment strategies may change the prognostic value of traditional prognostic variables in ovarian cancer. Some of these may serve as new prognostic markers. This study focuses on the evaluation of the significance of several putative clinical and biologic markers, for predicting outcome in patients with ovarian cancer. Consequently data acquisition and analysis has primary importance. The clinical information as well as molecular data was stored in a database. Univariate analysis technique used to identify the clinical and biologic significance of each marker, specifically determining if the presence or absence of each marker was associated with overall survival and RFS. Putative prognostic variables were analyzed using Kaplan-Meier survival analysis for their prognostic significance (Lee, 1992a). Any putative prognostic marker was also evaluated by multivariate analysis to determine if its predictive value was independent from that of the traditional markers according to the recommendation of AJCC. Multivariate analysis to evaluate the new marker(s) identified by univariate analysis for their clinical significance was assessed by the Cox proportional method (Lee, 1992b). Because of the wide array of novel potential markers for ovarian cancer, it is necessary to formulate a conceptual model of the disease

to aid variable selection and ultimate analysis. We have constructed a hypothetical model to aid evaluation of data collected in this study.

Hypothetical model predicting disease aggressiveness

A Theoretical model of cancer progression and outcome is proposed below:

Outcome = Extent of disease + Tumor Aggressiveness Factors + Patient factors + Drug resistance - completeness of excision.

We predict that the new biological markers will only be useful if they are a direct measure of tumor aggressiveness, drug resistance, or disease volume.

This study also concentrated on new techniques and markers that could easily be placed in clinical practice in routine hospital diagnostic laboratories.

To develop a prognostic model described above this investigation focuses on techniques that could provide quantitative and semi-quantitative data in an effort to produce a reproducible final model. Moreover, specific knowledge of the tumor properties and chemistry may aid targeted drug selection in the future. For example, providing prognostic information in the case of a patient at an early stage of tumor would be highly desirable, while the same information would be of no value in the case of a patient undergoing palliative care. Flow cytometry is an example of a quantitative technology in which presence of aneuploid populations and the proliferation fraction can be rapidly, efficiently, and accurately determined unlike grading which can be non-specific. Flow cytometric DNA analysis of fixed tissue was first introduced by Hedley, of the Ludwig Institute in Sidney, Australia, 1983 (Hedley et al., 1983). Flow cytometric analysis of DNA and proliferative activity have been demonstrated as a markers of tumor aggressiveness in ovarian cancer. Although there are conflicting results of the independent

prognostic significance of S-phase fraction, however, ploidy has been demonstrated as a marker of independent prognostic significance (reviewed in Braly, 1992).

The aim of immunoexpression studies was to assess whether the expression of certain markers, are predictive of disease behavior in patients with ovarian carcinoma. Overexpression of protein products from oncogenes like ErbB-2 and Cyclin D1 and tumor suppressor genes including P53 have previously been correlated with an unfavorable outcome in ovarian cancer patients (Slamon et al., 1989, Worsley et al.,1997, and Boente et al., 1994)

Oncogenes are activated in cancer due to qualitative as well as quantitative changes resulting in increased expression of the protein or in the production of an altered protein with enhanced activity. Qualitative changes in gene are characterized by point mutation and rearrangements in the gene including structural alterations, such as translocations, inversions, deletions, alternative splicing and insertions. Quantitative changes include gene amplification, overexpression by increased transcription or translation, and stabilization of either mRNA or protein. Amplification of a gene in DNA can be easily assessed by Southern blot or dot blot hybridization depending on the source of the tumor tissue fresh or formalin fixed, PET. The advantages of using dot blot hybridization is that a number of specimens can be quantitated simultaneously on one nitrocellulose membrane. ErbB-2 amplification in ovarian carcinoma has been correlated with adverse outcome occurs in 25-30% of cases (Slamon et al., 1989).

There appears to be several fundamental characteristics that are features of all cancers, these are proliferative capacity, alterations in apoptosis/senescence capability (immortality), genomic instability, and drug resistance. Because these features are the

embodiment of malignancy, every cancer will exhibit some or all of these fundamental characteristics to some degree. The development of these characteristics appear to be produced by underlying genomic mutations. Each cancer will differ in the severity and significance of each of these components of malignancy resulting in different clinical behavior. Hence, prognosis could be better predicted by accurately quantifying all aspects of the malignant cells. While these broad categories of cellular alterations are recognized, it is not always possible to measure them directly. One approach is to use surrogate variables such as aneuploidy as a marker of genomic instability. Many of the molecular markers under study are related to gene products of genes controlling aspects these broad categories. For example, the CDK/RB pathway control genes may be expected to have direct bearing on the proliferative capacity of the carcinomas, P53 is both a proliferative modifier and involved in maintaining genomic stability, inducing apoptosis. Cells with defective apoptosis may also be drug resistant. Consequently the measurement of a specific marker may not be "pure" and results appear to be contradictory on initial analysis.

In clinical oncology there are a number of advantages in using PET for retrospective studies because of the huge existing collections of PET in many pathology laboratories (Coon et al., 1986). The recent adaptation of many new technologies now enables numerous genetic markers to be analyzed in archival PET greatly enhancing this value for understanding the role of molecular alterations in human cancers and the direct relationship of many to the clinical behavior of the malignancy. Therefore statistically accurate assessments of long-term prognostic significance of each of the marker can be easily determined without the long study periods of prospective analysis. Secondly,

molecular assessment of many rare and unusual tumors are not practical with prospective analysis using fresh tissue requiring many years to accumulate sufficient numbers of rare cases.

Chapter 3

3.0 Materials and methods

Figure 2 represents a schematic view of the work flow and numbers of cases available for each component of the analysis in this study.

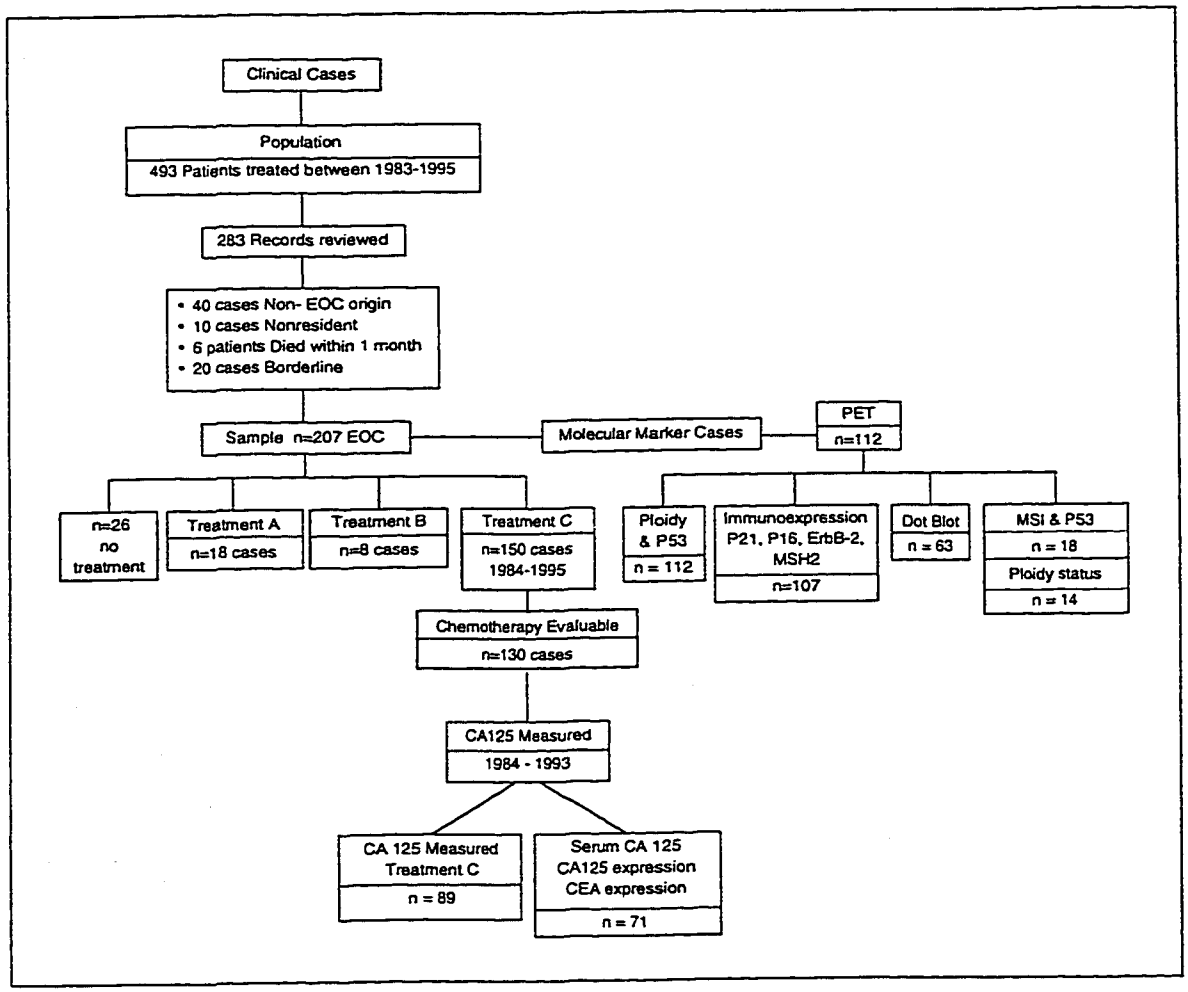

Figure 2: A schematic summary of an ovarian cancer retrospective study that included clinical and molecular prognostic markers. Treatment A: Melphalan, B: Radiation and/or chemotherapy, C: Platinum based regimens.

3.1 Collection of clinical information in the current investigation

Saskatchewan with a population of about 1 million, has a centralized cancer registry system with registration of over 90% of the cancer cases occurring in the province. Cancer treatments are centralized at two cancer clinics in the province, one of which is the Saskatoon Cancer Centre, Saskatoon. This clinic, serves Northern Saskatchewan (North of Davidson) and covers approximately half the population. All cases of ovarian cancer occurring between 1983-1995 were identified using the Saskatchewan Cancer Agency Registry. (The years between 1983 and 1995 were chosen because treatment in this period was with contemporary platinum containing regimens and it allowed at least a 5 year follow-up outcome analysis to be performed).

Overall, 283 cases were reviewed out of a total of 493 cases registered, treated at the Saskatoon Cancer Centre, between the years 1983-1995. Seventy six cases were excluded for one of the following reasons:

Non-resident visiting Saskatchewan (10 cases); death occurred within one month of diagnosis (6 patients - since the time is insufficient to respond to treatment (surgery and/or chemotherapy), borderline tumors (20 cases); non-epithelial cancer (40 cases).

Two hundred and seven epithelial ovarian carcinomas cases (about 73%) were randomly chosen from this record and effort was made to include cases from each stage category. Detailed clinical data was collected for each case from the cancer agency records (Table 3). The age at diagnosis, body surface area, and the largest tumor size at laparotomy, were the continuous variables which were divided into two groups depending on the mean value for statistical analysis. Data on initial surgery includes: BSO/USO, TAH. Residual disease was defined as a) no evidence of disease (NED) (clinical and/or

surgical absence of disease), b) minimal residual disease (≤ 1cm remaining after surgery), and c) gross residual disease (> 1cm remaining after surgery). Some categories were combined: Stage I - II were coded as early stage (ES) and Stage III - IV were coded as advanced stage (AS), and, Performance status (ECOG scale) 0 and 1 - 4 were coded as all others. Performance status (ECOG) scale coding scheme is described as follows:

Grade

0 Fully active and capable of performing all pre-disease activities.

1 Restricted in physically strenuous activities but ambulatory.

2 Ambulatory and capable of self-care but unable to carry out any work activities.

3 Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.

4 Completely disabled, cannot carry on self-care and totally confined to chair or bed.

Table 3: Patient clinical information collected by a review of the medical record.

Type of surgery	BSO, USO, TAH, Omentectomy, Other pelvic pathology.
Clinical factors	Ascites, residual disease(NED, ≤1 cm, > 1 cm), performance status, CA125.
Tumor characteristics	Histologic type, tumor size, stage, grade, other pelvic pathology.
Other type of treatment	Chemotherapeutic agents and/or radiation therapy.

3.2 Collection of serum CA 125 level data

Data was found in the charts from the Abbott AxSYM® CA 125 assays done at the Pasqua general hospital in Regina, using OC 125 antibody with a kit from Centocor Inc. CA 125 level were coded as either as base line value: 0-35 u/ml or elevated levels: > 35 u/ml.

3.3 Retrieval of paraffin embedded tissue blocks from the different hospitals

Slides were reviewed to select blocks containing optimal and representative portions of neoplasm. In addition, "normal" tissue blocks were also selected by identifying blocks without any evidence of malignancy. These included fallopian tubes, cervix, and uterus. The pathology accession number was used to retrieve the PET blocks in the patients diagnosed with EOC in Saskatchewan. Patients were randomly selected, diagnosed with ES disease as well as some with AS disease, and the PET blocks were retrieved from the various hospitals in Saskatchewan.

3.4 Extraction of DNA from PET blocks

DNA was extracted according Goelz method (Goelz et al., 1985) with few modifications. The excess paraffin was cut off from the blocks with a razor blade and the tissue specimen was soaked in xylene for 48 hours. The blocks were sectioned initially and stained for haematoxylin and eosin (H&E) for further selecting blocks containing maximum tumor cells for the molecular analyses. The deparaffinized tissue was rehydrated by placing sequentially in absolute alcohol for 2 hours, 95% ethanol for 2 hours, and 70% alcohol for 2 hours. After decanting the 70% alcohol the tissue was placed in nanopure water to

remove any remaining alcohol. The tissue was then minced and transferred to (15 ml) polypropylene tubes with Tris-EDTA (TE), pH 9 (~5 ml). The tissue was then homogenized and centrifuged at 3000 rpm for 10 minutes. The supernatant was removed and sodium dodecyl sulphate (SDS) (1%) and proteinase K (250 µg/ml) was added. The tubes were then incubated overnight. An equal volume of phenol/chloroform/ isoamylalcohol (25:24:1) was added to the tubes following incubation. The tubes were rotated on an autoshaker for 10 minutes and centrifuged at 1000 rpm for 10 minutes. After three phenol/cholorcform/isoamyalcohol extractions and one choloroform /isoamylalcohol extraction to the aqueous phase, 3M sodium acetate (pH 5.2 to obtain a final concentration of 0.3M sodium acetate) and twice the volume of absolute alcohol was added. The tubes were then cooled to -20°C overnight to allow the DNA to precipitate. The tubes were then spun at 15, 000 rpm for 10 minutes to pellet the DNA and the supernatant was discarded. To the DNA pellet an equal volume of 70% alcohol was added to remove any dissolved salts and the tubes were spun at 15, 000 rpm for 10 minutes. The supernatant was removed and the DNA pellet was allowed to air dry at room temperature in a fume hood. DNA was resuspended in DNA suspension buffer TE. DNA obtained was quantified spectrophotometrically (Phillips PSPU 8700, UV\VIS). The readings obtained at 260 nm was used to calculate the DNA concentration. 10µg of the recovered DNA by this procedure was electrophoresed on 1.2% agarose gel to observe the quality of the DNA.

3.5 Immunohistochemical detection of protein expression on tumor paraffin embedded tissue blocks from ovarian cancer patients

Appropriate PET blocks containing over 70% neoplastic tissue were selected for the study. 5μm sections of each case were cut and stained with the various antibodies as indicated in Table 4. Immunostaining staining was performed by the Labeled Streptavidin-Biotin (LSAB) method using a kit from Dako, Carpinteria, CA). The paraffin sections were deparaffinized in xylene and rehydrated through graded alcohols. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide in methyl alcohol for 10 minutes. Each section was treated by microwave in 0.01 M citrate buffer (pH 6.0 for staining with the exception of two, ErbB-2 and CEA) for 12 minutes. Nonspecific reactivities were suppressed with DAKO Universal blocker (Carpinteria, CA) for 10 minutes. The sections were then incubated with specific primary antibodies (dilutions as shown in Table 4) overnight at 4° C. The slides were then washed with PBS, link reagent was applied for 30 minutes at room temperature, followed by Streptavidin reagent for 30 minutes also at room temperature. After washing with PBS, the slides were preincubated with 0.03% 3'-3' diaminobenzidine solution for 3 minutes and then incubated with hydrogen peroxide conjugated 3'-3' diaminobenzidine solution for 5 minutes. The reaction product was then enhanced with 2% copper sulphate solution for 5 minutes. Counterstaining was performed lightly with Harris's hematoxylin and blue in warm tap water before mounting.

Table 4: Antibodies used in immunohistochemical analyses.

Antibody	Species	Type	Clone #	Source	Dilution
P53	Mouse	M	D07	Dako, Carpinteria, CA	1/50*
$P21^{WAF1/CIP1}$	Mouse	M	EA 10	Oncogene science, Cambridge, MA	1/10*
P16INK4A	Rabbit	P	G175-405, 13251A	Pharmingen, Mississauga, ON	1/500*
Erb-B2	Rabbit	P	A 485	Dako, Carpinteria, CA	1/400
MSH-2	Mouse	M	FE11	Oncogene science, Cambridge, MA	1/20*
CA 125	Mouse	M	M11	Dako, Carpinteria, CA	1/20*
CEA	Rabbit	P	115	Dako, Carpinteria, CA	1/6000

* Antigen retrieval, M: monoclonal, P: polyclonal.

The immunoreactivity was semiquantitatively evaluated by two observers (Advikolanu and Magliocco) and scored using the following system as shown in Table 5 for all proteins except of $P21^{WAF1/CIP1}$. The cases with scores 0 and 1 were considered to be negative for immunomarker and the remaining scores, 2, 3 and 4 were considered to be overexpressors for the particular protein. For $P21^{WAF1/CIP1}$, positive nuclei were counted in high power (600x) in five fields. A case with any positive nuclei was categorized as a positive.

Table 5: Scoring system applied for measurement of protein expression in the immunoreactivity studies.

% Of Malignant Cells Staining positive	Score
0-10	0
10-25	1
25-50	2
50-75	3
75-100	4

3.6 Dot blot hybridization studies

Sixty-three cases of primary ovarian cancer were randomly selected for retrospective evaluation of the copy number of the selected G1-S check point genes viz., Rb-1, Cyclin D1, $p16^{INK4A}$ and CDK4 by dot blot analysis (Figure 1).

Representative PET blocks were obtained for each case entered into the study. Care was taken to select blocks that contained predominantly malignant cells with minimal stroma or areas of necrosis. DNA was extracted from PET by an organic solvent method and quantified spectrophotometrically as described in section 3.4 and was redissolved in TE buffer (10mM Tris & 1M EDTA, pH 7.4) and denatured in 0.25 N sodium hydroxide for 10 minutes. 10 and 12.5 µg of DNA per dot was blotted onto Sure blot (Oncor MD) using the Minifold ™ filtration manifold (Schleicher & Schuell, Inc.). The DNA samples were allowed to remain on the Sure Blot membrane and filter pad (Whatmann 3 MM) without any suction for 30 minutes. After 30 minutes a slight suction was applied to the manifold for approximately 30 seconds. The membrane was removed and baked for one hour at 80^0 C. The following were the details of the vector, restriction enzyme, insert size and source of the probe(American type tissue culture{ATCC}, and Beach laboratories), Table 6.

Table 6: Hybridization probe insert size, restriction enzyme, vector and source of the probes.

Probe cDNA	Insert size	Restriction enzyme	Vector	Source
Rb-1	1400	Hind III	pBR22	ATCC
G6PD	1500	EcoRI	pUC18	ATCC
$p16^{INK4A}$	960	EcoRI and Xho I	pBlue Script	ATCC
Cyclin D1	1300	EcoRI	pUC118	ATCC
CDK4	950	NCO and SmaI	pBlue Script	Beach laboratories

All the probes were labeled by random priming with a specific activity of 1-2x10^9 dpm/ug using [α-^{32}P] dCTP at 3000 Ci/mmol. The membranes were hybridized with random-primed ^{32}P labeled probe overnight at 42^0 C using oligolabelling kit from Pharmacia. Following hybridization the unbound probe was washed off at room temperature (3 x 15 minute each) and the fourth wash was at 52 ^{0}C for one hour with 0.1% SSC and 0.1% SDS. The membranes were then exposed to X-ray film (Kodak, X-Omat AR) with an intensifying screen at -80 ^{0}C for 12-65 hours. Membranes were stripped and rehybridized with each labeled probe and also labeled with G6PD probe (located on chromosome X q28) as a control.

3.7 Flow cytometry for assessment of ploidy status

The tissue for flow cytometric evaluation was prepared by a method described in literature (Geissel and Griffin, 1994). The PET blocks were cut into 2 x 40μm sections and placed in 10ml glass centrifuge tubes, deparaffinized by treating with xylene, 3 ml, for 10 minutes two times.

Overall one hundred twelve cases were analyzed for DNA content. Normal tissue (PET), nonmalignant tissue were used as external ploidy standard for diploid "normal" population. After aspirating the xylene, the samples were rehydrated by two treatments with 100% ethanol, 3 ml, 10 minutes each. Followed by decreasing concentrations of ethanol (95%, 80%, 70%, and 50%), 3 ml, for 10 minutes each, and ending with deionised water, 3ml, overnight at 4° C. The isolation of nuclei was performed by adding 1 ml of

protease solution to the glass tube and incubating at 37° C in water bath for 1-2 hours. The samples were then removed from the incubator and kept on a shaker for 30 minutes. The samples were then washed with phosphate buffered saline (PBS), pH 7.4, ~ 6 ml, the section was removed and sample was centrifuged at 3,000 rpm for 10 minutes. After the supernatant was aspirated another 1 ml of PBS, pH 7.4, ~ 6 ml was added to the samples. The nuclei were resuspended by syringing the pellet thoroughly with a 22-gauge spinal tap needle attached to a 3-ml syringe. The nuclei were counted in a hemacytometer and about 50, 000 - 2 x 10^9 cells ml^{-1} were obtained. The nuclei were then centrifuged at 3000 rpm, at 4° C, for 10 minutes. The supernatant was aspirated and the nuclei were resuspended by syringing with a spinal tap needle. Triton-PBA, 1 ml/10^6 cells was added to each sample, vortexed, and kept on ice for 3 minutes. The samples were centrifuged, and the supernatant was aspirated and syringed. To the samples RNAse solution (500 units/ml) was added, 1ml/10^6 cells was added and the samples were incubated at 37° C in a water bath for 30 minutes. Following which centrifuged, aspirated and syringed, propidium iodide solution (0.1 mg/ml) per 1ml/10^6 cells was added. The tubes were then vortexed, aluminium foil was wrapped around each tube and stored at 4° C overnight. After the nuclear DNA content was filtered through 35-µm nylon mesh, it was measured on a Coulter Epics XL flow cytometer.

3.8 Assessment of microsatellite instability (MSI)

DNA from PET was isolated using a Qiagen™ DNA isolation kit (Qiagen, Inc., P/N 29304, Mississauga, ON) and estimation of the DNA concentration was determined with

spectrophotometrically. The PCR mixes from the microsatellite RER/LOH kit (Perkin Elmer Applied Biosystems, A division of Perkin Elmer, P/N K0015, Foster city, CA) was used to access 9 microsatellite loci for RER and MIN. The PCR reactions for the fluorescent markers were done in total volume of 10μl and included PCR buffer, $MgCl_2$, deoxyribose nucleotide triphosphates (dNTPs), 12.5 ng genomic DNA, 0.5 U AmpliTaq Gold DNA polymerase, and the locus specific primer sets (one of them fluorescently labeled).

The RER/LOH assay was performed as described below for nine loci and the primers were also obtained from Perkin Elmer (PE). Markers we used in this study to determine RER/LOH for DNA isolated from PET are given in Table 7.

Table 7: The microsatellite assay markers (from Perkin Elmer) for DNA from ovarian cancer patient specimens.

Marker	Chr.	Max. Heter.[a,b]	Size (bp)	Primer dye (color)	Gene near marker
NM23	17q21	0.7	75-105	FAM(blue)	NM23
D18S35	18q21	0.703	88-126	TET(green)	DCC
TP53-Di[c]	17p13	0.694	97-132	HEX(yellow)	P53
D5S346	5q21	0.82	98-125	FAM(blue)	APC
TP53-Penta[d]	17p13	unknown	140-169	FAM(blue)	P53
D2S123	2p16	0.773	140-180	TET(green)	MSH2
D1S2883	1q24	0.754	170-199	FAM(blue)	HPC1
D3S1611	3p22	0.664	180-200	TET(green)	MLH1
D7S501	7q31	0.818	200-240	TET(green)	MET

a: Maximum heterozygosity, b: The maximum heterozygosity and amplicon size ranges for many of the markers were obtained from the Genome Database http://gdb.org/. The amplicon size may be slightly different from published values depending upon the location of the primers or the population observed, c: Dinucleotide and d: Pentanucleotide.

The normal and tumor genomic DNA was diluted to a concentration of 10ng/μl using PCR grade water. A master mix of the reagents for PCR was prepared as follows. Following were the dilution volumes for a 12x master mixes in a total volume of 30μl: 13.3 μl of dH_20, 1.2 μl of AmpliTaq Gold (5U/μl) and 15 μl normal or tumor genomic DNA (10ng/μl). 2.5 μl of genomic DNA/Taq Gold master mix was added to the PCR mixes in each of 0.2ml microAmp tubes for each of the nine loci. The reaction tubes were spinned in a centrifuge for about 20 seconds at approximately 150 x g.

The thermal cycling conditions as developed by PE Applied Biosystems were utilized for PCR reaction on a Perkin Elmer 2400 Cetus thermal cycler and MJ Research, PTC 200, Peltier thermal cycler. The PCR thermal cycling steps are described below.

Initial denaturation and Taq Gold activation, 95°C 10 minutes linked to: 45 cycles of 96°C 10 seconds, 55°C 30 seconds, 70°C 3 minutes linked to final extension (to bring a reaction to completion) 70°C 30 minutes linked to: 4° C soak.

Since there are variations in dye intensities and amplification efficiencies (particularly with paraffin DNA), it was recommended by the manufacturer that different volumes of amplified products be pooled prior to Genescan analysis. The following were the pooling volumes for each fluorescent amplicon: 1µl of each primer product except for primer 4, 1 5µl PCR product were pooled for normal and tumor pair for each patient specimen because of interference between amplimers of similar colors and sizes. The fluorescent amplicon primer PCR product 1, 5, 8-10 were pooled together whereas the primer amplicons PCR product 2-4, 6 and 7 were pooled in another sample tube. A 1x mix of 12µl deionized formamide and 0.5µl TAMRA-350 was added to 0.5µl of each pooled PCR product in a sample tube with a septum. The sample tubes were denatured at 95° C for 2 min. and kept on ice for 5 min. The sample tubes were then placed in a autosampler tray on a 310 PE Genescanner. The sample were run at 15 kilo volt (KV) for 24 min. using a electrophoresis tube (5-47 cm x 5 cm, part No. 402839, PE, Foster city, CA, USA) and on a polymer performance optimized from PE at 60° C. The injection time for each sample varied between 1 and 5 sec. The Genotyper™ was used to interpret data on MSI and LOH. The Genotyper software filters the peak labels, thereby removing the labels from stutter peaks and leaves size labels only on the allele or the RER peak at each marker for normal and tumor specimen analyzed

3.9 Statistical analysis in the current investigation

The age at diagnosis, body surface area, and the largest tumor size at laparotomy were the continuous variables and were arbitrarily divided into two groups depending on the mean value for analysis. Some categories were combined: Stage I and II - ES and Stage III and IV - AS. CA 125 levels were coded as either as baseline value: 0-35u/ml or elevated levels: >35u/ml.

Patient status at the end point (September, 1997) was recorded - a) alive with disease, b) alive without disease, c) dead of disease, and d) dead of other causes. Clinical factors were evaluated for their value as a predictive marker for the following two outcome events: a) Survival (calculated from the date of pathological diagnosis to the date of death or last follow-up). b) RFS (calculated from the date of diagnosis to the date of first relapse or the last follow-up where relapse had not occurred). A detailed data sheet which was developed to review the medical records was used to record the clinical data (appendix A).

Score 0 and 1 was considered to be immunonegative and score 2, 3, and 4 was considered to be positive for p53, MSH-2, $P16^{INK4A}$, erbB-2 immunoreactivity. Score 0 was considered to be immunonegative and the remaining 1-4 as immunopositive for $p21^{WAF1/CIP1}$, CA 125, and CEA immunoreactivity.

Results were classified as an amplification, deletion or no change for the genes examined by dot blot hybridization.

DNA content by flow cytometry was classified into either diploid or aneuploid cell populations from the histograms obtained. Since there were only two cases with tetraploid peaks, these cases were included in the aneuploid category for analysis.

The data acquired for MSI analysis in ovarian cancer cases was analyzed by Genotyper software at the end of each run. Each fluorescent peak was quantitated to give the size in bp and height of each fluorescent product. For each marker the allele LOH ratios were calculated as described in Canzian et al., 1996. The value for peak heights of the two alleles in the paired normal and tumor specimens was used to calculate a ratio and determine if there was allele loss. The ratios of the alleles was determined for each paired normal and tumor sample and then divided by the tumor ratio, i.e.

$$\text{LOH} = \frac{\text{Height of normal allele 1/ Height of normal allele 2}}{\text{Height of tumor allele 1/Height of tumor allele 2}}$$

LOH was considered to be positive if the ratio of </= 0.5 or >1.5 was obtained.

For RER determination, a specimen which exhibited multiple peaks in the tumor sample, as compared with the normal pair, was classified as RER+ for the particular marker. Samples which were found to be homozygous or an RER+, such loci were considered uninformative for LOH analysis.

The molecular factors were evaluated for their value as a predictive marker for survival, RFS, and adverse outcome event. A patients death within five years from diagnosis was defined as an adverse outcome event.

Data on all the patients in the study and the related molecular parameters investigated were entered onto a common data base using SPSS for Windows Version 8.0 and subsequently analyzed with the same statistical software. Kaplan-Meier product limit method (Lee, 1992a) was used to describe survival distributions and observed differences in survival were identified by the logrank test using a significance level of *0.05*. Fisher's exact test was used to determine the association between categorical variables. The Cox's

proportional hazard models were used to identify independent prognostic factors for survival and time to relapse. The multivariate model was built using forward selection of variables (Lee, 1992b).

Chapter 4

4.0 Results of patient, treatment, tumor, and tumor specific factors in ovarian cancer patients

A summary of the patient, treatment, tumor and tumor specific factors, related to the patient reviewed of Saskatchewan Cancer Agency, are shown in Table 3. The mean age of the patients at the time of diagnosis was 57.5 years (standard deviation: 13.58 years, range 18 - 84 years). Follow-up of these patients extended up to 13 years with a median follow-up of 33 months. The overall 5 year survival rate was 45% for all stages in the current investigation. The overall median length of survival and RFS was 35 and 20 months respectively.

One hundred and thirteen patients died due to ovarian cancer; four patients died due to other causes. However these cases had previously relapsed with ovarian cancer, and, five deaths have been attributed to other causes.

There were seventy-seven cases who were alive and relapse free, and eight cases who were living with recurrent disease at the end of 56 and 28 median months of survival. One hundred and eight patients (52%) had AS disease and eighty-six patients (41%) at the time of diagnosis had ES disease and in thirteen patients (6%) the data on stage was not available. Serous cystadenocarcinoma (42%) was the most common histologic type, followed by endometrioid adenocarcinoma (18%), undifferentiated carcinoma (17%), mucinous cystadenocarcinoma (12%), and clear cell carcinoma (7%). There were 120 patients (58%) who had other coexisting pelvic pathology (leiomyoma, fibroma) at the time of laparotomy.

Eighteen variables (age at the time of pathological diagnosis, body surface area, performance status - ECOG scale, tumor size at laparotomy, ascites, adhesion, rupture, stage, initial surgery - BSO or USO, bilateral ovarian involvement, TAH, omentectomy, other coexistent pelvic pathology, residual disease (NED, minimal, gross), peritoneal cytology, histologic type, grade, and chemotherapy (platinum regimen), were analyzed for relationship with survival and RFS using the log rank test.

There were 152 patients treated with platinum based combination regimens, 18 patients with melphalan and 34 patients did not receive any type of therapy. Four patients received radiation therapy alone and four patients received chemotherapy as well as radiation therapy. In the current study there were 134 cases (88%) of evaluable platinum based combination therapy - Cisplatinum/Carboplatin and Cyclophosphamide. (Patients were considered to be evaluable if they received more than two cycles of chemotherapy).

An individuals record was taken of the dosage of each chemotherapy drug, the duration of treatment and body surface area. Relative dose intensity (RDI) was calculated as delivered dose intensity (DDI) divided by projected dose intensity (PDI) (Longo et al., 1991). PDI is the total amount of drugs scheduled divided by the projected time schedule of the entire treatment. DDI represents the total amount of drug actually received divided by the time taken for therapy. The RDI, in these patients treated with platinum agent (Cisplatin or Carboplatin) and Cyclophosphamide ranged from 33 to 100%. The response to treatments was assessed by the two outcome measurements, survival and RFS in the one hundred and thirty four patients, after dividing into two categories based on RDI: $\leq$ 80% and >80-100%.

Patients (20, 15%) with a RDI between ≤ 80% had a shorter median time of survival, 9 months than the patients (114, 85%) receiving greater than >80-100% (46 months, $p = 0.0005$) by log rank statistics. Patients receiving between >80-100% of the RDI had a statistically significant difference ($p = 0.0001$) in median RFS time of 25 months in contrast to the other group (6 months).

By univariate analysis of seventeen variables, those with mucinous carcinoma had the best prognosis, those with undifferentiated carcinoma had the poorest and endometrioid, serous, and clear cell carcinomas had intermediate prognosis in decreasing order both for survival and RFS. The differences in survival time and RFS for ES and AS disease was significant as was ascites, residual disease, tumor size at the time of laparotomy, and grade.

The factors that were significantly related to relative risk of death and RFS are shown in Table 8 and 9. Multivariate analysis to identify independent factors that were significantly correlated to survival identified stage (Fig. 3) and residual disease (Fig. 4) were the independent prognostic indicators in one hundred and seventy three cases that were available for analysis. Furthermore, stage (Fig. 5), and residual disease (Fig. 6), were the two independent prognostic factors in the Cox's proportional hazard model, when the outcome measurement was RFS. Other pelvic pathology, peritoneal cytology, and performance status were not included in the multivariate model for survival and RFS since data was available on 190, 110 and 81 cases respectively.

Table 8: Relative risk (univariate) of factors related to disease specific factors and overall survival in ovarian carcinoma patients.

Factors	N	Survival	RR[1]	95%CI	P value
Stage					<0.001
ES	87	116[3]	1	-	
AS	108	25	7.85	4.45, 13.84	
Residual disease					<0.001
NED	69	118[3]	1	-	
Minimal	47	46	4.48	2.16, 9.30	
Gross	72	19	10.85	5.56, 21.16	
Ascites					<0.001
No	90	103[3]	1	-	
Yes	112	30	3.52	2.28, 5.43	
Peritoneal cytology					0.0001
Negative	64	134	1	-	
Positive	80	24	3.70	6.25, 2.22	
Grade					<0.001
1	41	109[3]	1	-	
2	52	110	2.43	1.11, 5.20	
3	94	35	4.27	2.12, 8.59	
Bilateral					0.0006
No	92	100[3]	1	-	
Yes	99	34	2.72	1.76, 4.20	
Histology					0.003
Serous	86	50	1	-	
Undifferentiated	35	23	2.04	1.27, 3.27	
Mucinous	25	110	0.54	0.26, 1.10	
Endometrioid	38	106	0.52	0.28, 0.97	
Clear cell	14	42	1.12	0.50, 2.48	
TAH					0.0009
Yes	121	65	1	-	
No	80	35	1.88	1.28, 2.70	
Performance status					0.001
0	57	53	1	-	
All others	26	24	2.68	1.47, 4.90	
Tumor size					0.004
≤ 12 cm	83	40	1	-	
>12 cm	108	106	0.55	0.36, 0.83	
Adhesion					0.004
No	36	110	1	-	
Yes	163	42	2.42	1.29, 4.53	
Other coexistent pelvic pathology					0.01
No	70	38	1	-	
Yes	120	65	0.61	0.41, 0.90	
Age					0.04
≤ 57 years	102	63	1	-	
>57 years	103	40	1.46	1.01, 2.12	
Omentectomy					0.09[2]
No	139	53	1	-	
Yes	65	48	1.48	0.48, 1.06	
BSO					0.14[2]
No	59	52	1	-	
Yes	142	42	1.34	0.9, 2.04	
Body surface area					0.70[2]
≤ 1.7 m^2	57	46	1	-	
>1.7m^2	111	44	1.08	0.72, 1.61	
Rupture					0.75[2]
No	154	50	1	-	
Yes	45	51	0.93	0.59, 1.46	

N: number of patients, survival: median survival in months, RR[1] : relative risk of death, [2] : not significant., [3]: Mean survival and CI: confidence interval.

Table 9: Relative risks (univariate) of factors related to relapse in ovarian carcinoma patients.

Factors	N	Relapse free survival	RR[1]	95% CI	P value
Stage					<0.001
ES	87	107[3]	1	-	
AS	108	10	7.5	4.59, 12.25	
Residual disease					<0.001
NED	69	113[3]	1	-	
Minimal	47	24	4.46	2.37, 8.39	
Gross	72	9.0	11.62	6.46, 20.90	
Ascites					<0.001
No	90	88[3]	1	-	
Yes	112	13	3.07	2.06, 4.58	
Peritoneal cytology					<0.001
Negative	64	90[3]	1	-	
Positive	80	11	3.70	2.32, 5.88	
Bilateral					<0.001
No	92	92[3]	1	-	
Yes	99	14	3.07	2.03, 4.65	
Grade					<0.001
1	41	107[3]	1	-	
2	52	34	2.50	1.20, 5.20	
3	94	19	4.67	2.40, 9.07	
Histology					<0.001
Serous	70	34	1	-	
Undifferentiated	35	9	1.83	0.17, 2.87	
Mucinous	25	33	0.44	0.22, 0.90	
Endometrioid	38	68	0.49	0.28, 0.87	
Clear cell	14	31	0.72	0.33, 1.59	
Tumor size					0.0005
≤ 12 cm	108	30	1	-	
>12 cm	83	80[3]	0.50	0.33, 0.74	
Performance status					0.001
0	57	39	1	-	
All others	26	11	2.44	1.38, 4.28	
Adhesion					0.001
No	36	29	1	-	
Yes	163	21	2.54	1.39, 4.61	
TAH					0.004
Yes	121	35	1	-	
No	80	11	1.66	1.16, 2.38	
Age					0.07[2]
≤ 57 years	102	39	1	-	
>57 years	105	21	1.37	0.96, 1.96	
Other coexistent pelvic pathology					0.07[2]
No	70	21	1	-	
Yes	120	34	0.71	0.48, 1.03	
Omentectomy					0.28[2]
No	65	21	1	-	
Yes	139	31	0.82	0.56, 1.18	
Body surface area					0.39[2]
≤1.7 m^2	57	20	1	-	
>1.7m^2	111	17	1.17	0.80, 1.71	
Rupture					0.43[2]
No	154	26	1	-	
Yes	45	28	1.17	0.78, 1.77	
BSO					0.45[2]
No	59	24	1	-	
Yes	142	28	0.86	0.58, 1.27	

N: number of patients, survival: median survival in months, RR[1] : relative risk of relapse, [2] : not significant, [3]: Mean survival and CI: confidence interval.

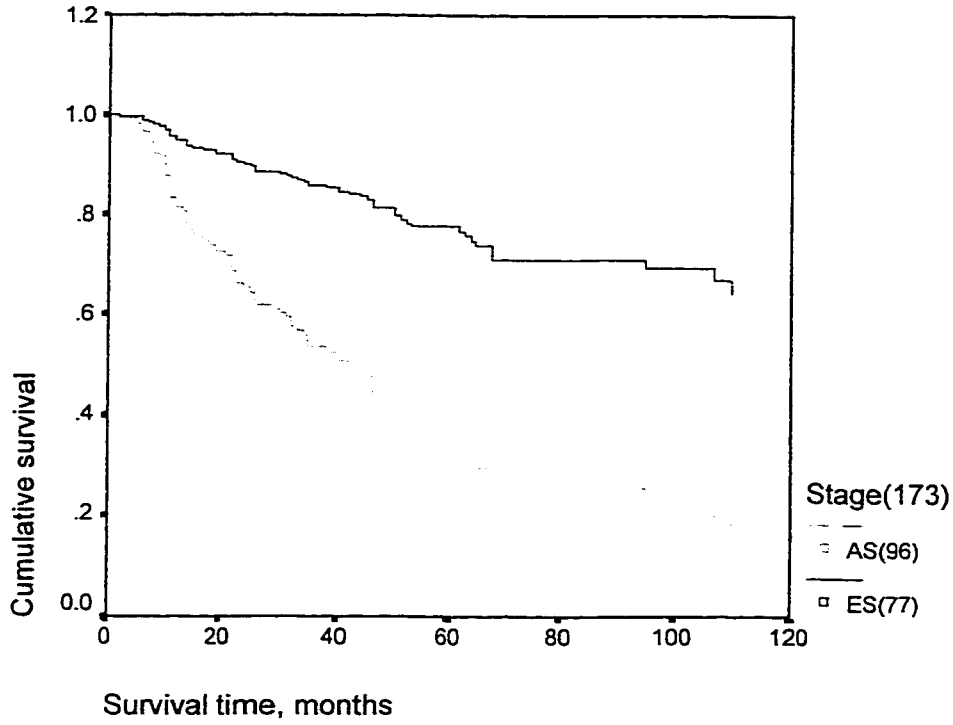

Figure 3: Survival curves of ovarian carcinoma patients diagnosed with early stage (77 cases) and advanced stage (96 cases).

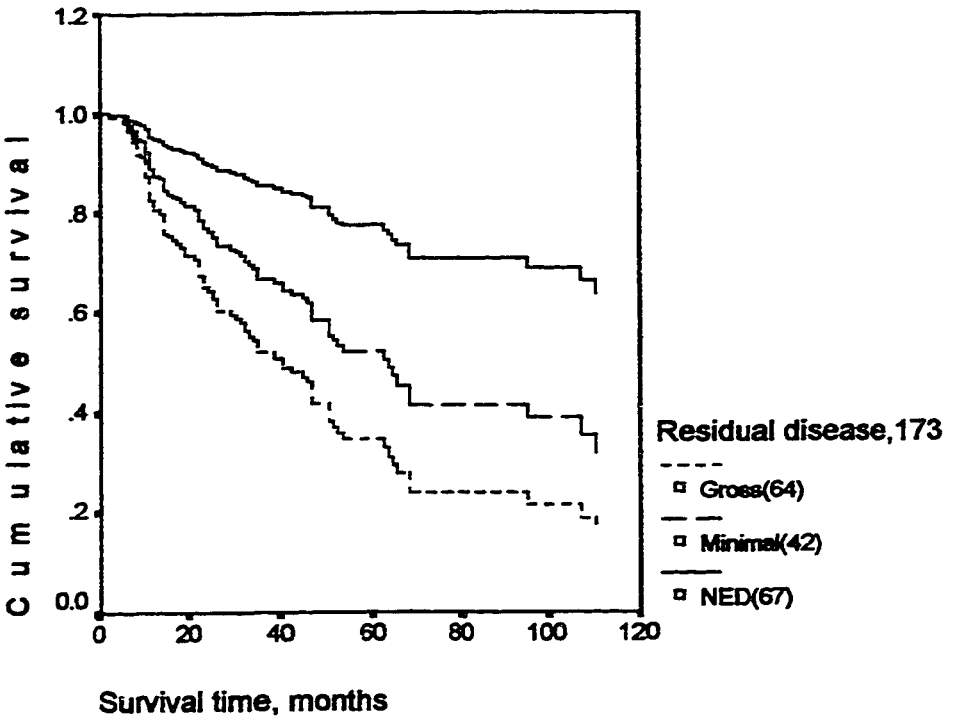

Figure 4: Survival curves of ovarian carcinoma patients with residual disease (NED,_, minimal,——, gross,_____).

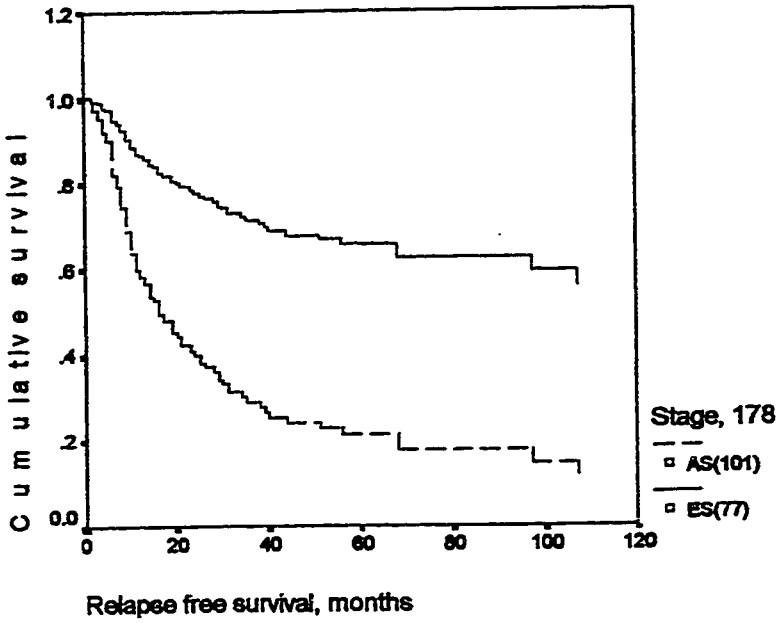

Figure 5: Relapse-free survival estimates of ovarian carcinoma patients diagnosed with early stage (ES, ——) or advanced stage (AS, ____).

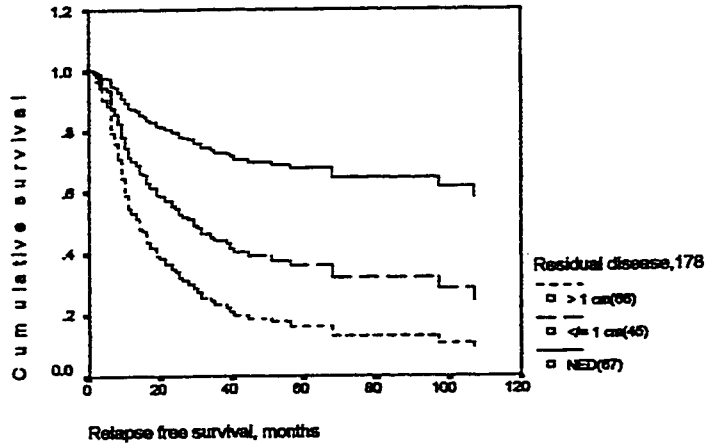

Figure 6: Relapse-free survival estimates of ovarian carcinoma patients with residual disease (NED, _, minimal, ——, gross, _____).

4.1 Evaluation of serum markers CA 125 and CEA in ovarian carcinoma patients

We observed four trends in longitudinal serum CA 125 levels. The four trends in CA 125 measurements observed as well as chemotherapy treatment periods are shown Figure 7. A

maximum of 37 data points and a minimum of 1 data points were collected for each case in the current study. CA 125 was measured during first line chemotherapy in 70 patients and in the remaining 19 cases the CA 125 levels were recorded during follow up/subsequent treatment periods.

Fig.8 a and b are each an example of CA 125 and CEA immunopositive tumors staining tumors of the ovary. CA 125 and CEA immunoreactivity evaluation in the PET were not correlated with outcome (Table 10). In Fig. 9 the immuno-overexpressors of tumor markers CA 125 and CEA can be seen in the different histologic types of EOC. Data in Table 11 indicates histologic type of EOCs and CA 125 serum levels along with the protein expression of the two markers, viz: CA 125 and CEA. 94% (28 out of 30 cases) of serous carcinomas reacted positively with antibody MI1 in contrast to 20% (1 out of 4) of the mucinous tumors. In the nonserous nonmucinous tumors 89% stained immunopositive (31 out of 35), Table 11.

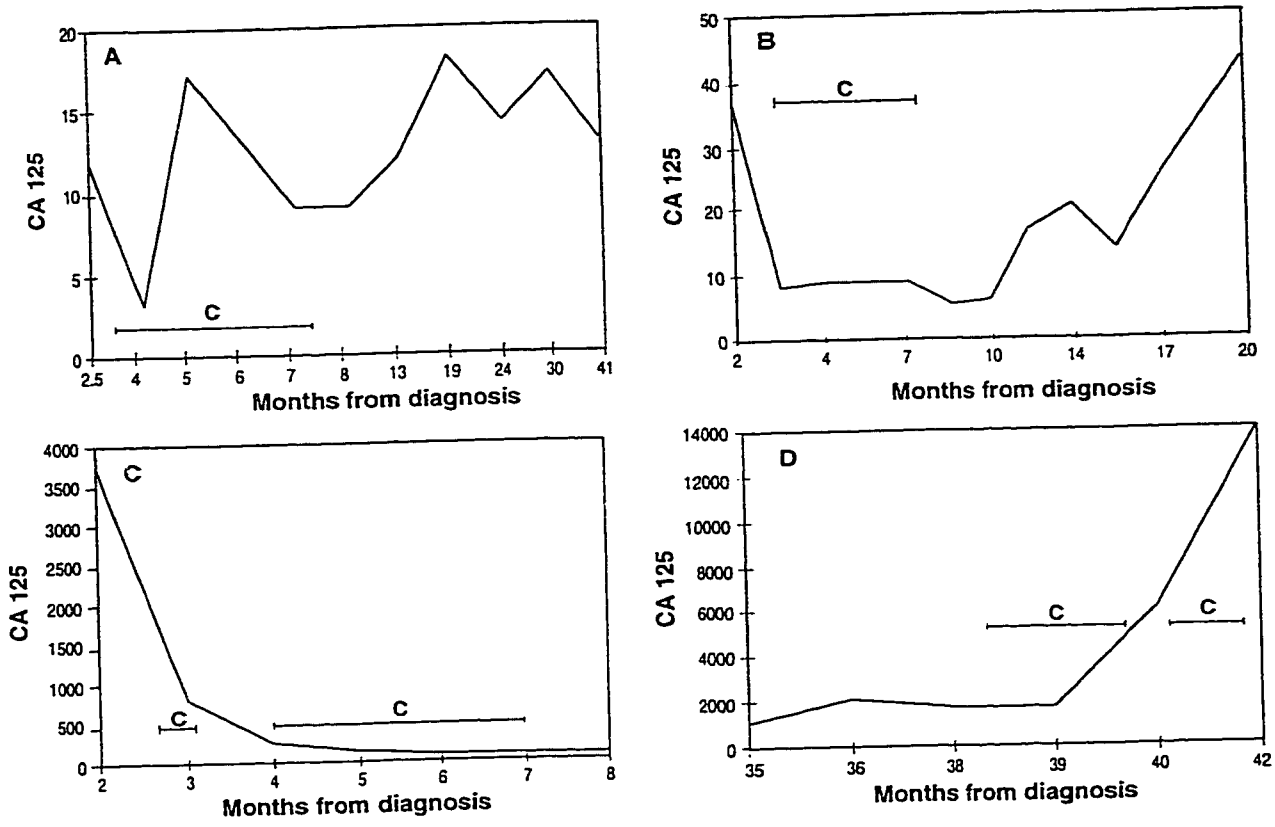

Figure 7: Trends of CA 125 levels observed and chemotherapy period in the ovarian cancer patients. A: CA 125, 0-35 units/ml, B: CA 125, 0-35 units/ml and increasing, C: CA 125, 35 units/ml and decreasing, and D: CA 125, > 35 units/ml and increasing. C = Chemotherapy period.

Figure 8a

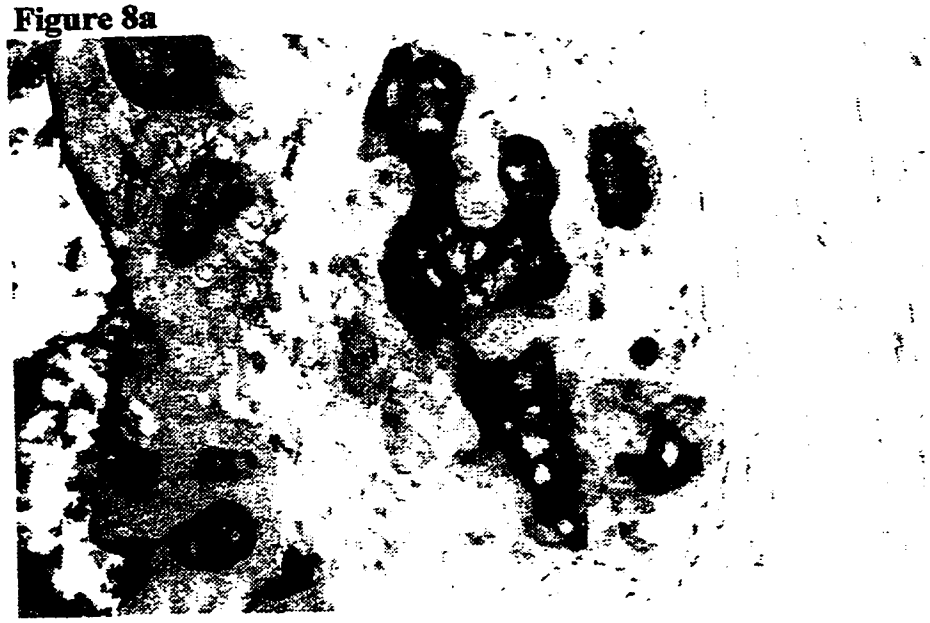

Figure 8b

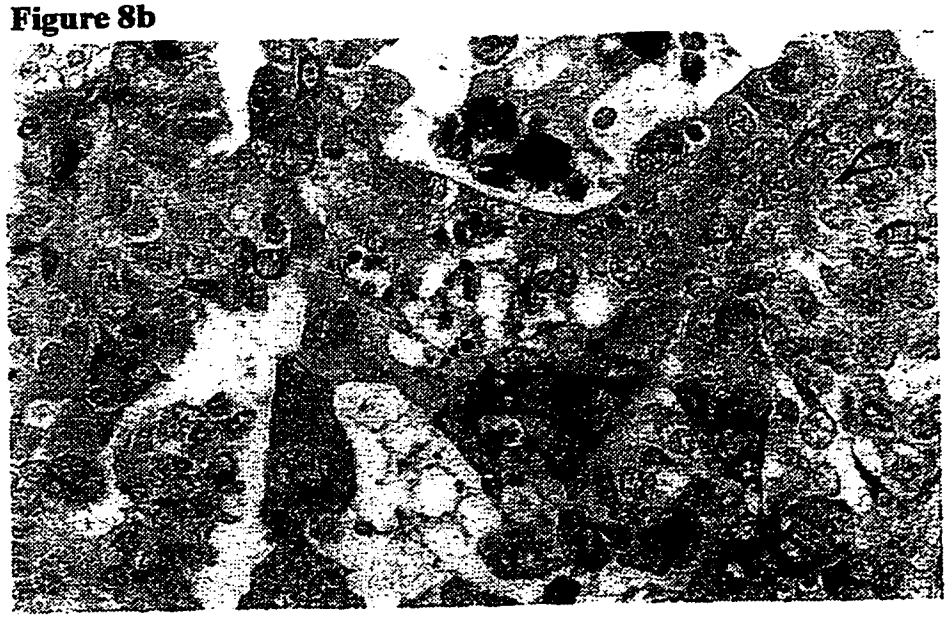

Figure 8a and b: CA 125 and CEA overexpression in paraffin embedded tissue from patients with ovarian carcinoma.

Table 10: CA125 serum levels and immunoreactivity of PET from ovarian carcinoma patients associated with survival and relapse-free survival.

Prognostic marker	Cases	Median survival months	RR of death	95% CI	P value	Median RFS months	RR of relapse	95% CI	P value
Serum CA 125	Total (89)								
0 - 35 u/ml	44	110	1	-	<0.001	97	1	-	<0.001
> 35 u/ml	45	23	5.86	1.03-33.08		11	7.15	3.76-13.60	
Immunostaining CA 125	Total (71)				0.24				0.78
Negative	11	25	*			14	1	-	
Positive	60	62	*			26	0.89	0.39-1.99	
CEA	Total (71)				0.74				0.47
Negative	43	50	1	-		25	1	-	
Positive	28	62	0.74	0.13-4.09		28	0.80	0.43-1.47	
Serum Immunopositive CA 125	Total (60)	110			<0.001				<0.001
0 - 35 u/ml	29	25	1	-		70	1	-	
> 35 u/ml	31		5.22	0.90-30.10		11	7.59	3.38-17.04	
Immunonegative CA 125	Total (11)				0.03				0.05
0 - 35 u/ml	8	50	¥			35	1	-	
> 35 u/ml	3	7	¥			10	4.81	0.80-28.91	

RFS: relapse free survival, *: reliable estimates could not be obtained due to few failure events, ¥: sample size is too small for cox regression analysis.

Table 11: Histological type and immunostaining of PET stained with CA125 and, CEA antibody, and serum levels of CA125.

Serum markers	Histology at laparotomy, total cases (%)				
	Serous, n=30	Endometrioid, n=17	Clear cell, n=4	Poorly, n=14	Mucinous, n=5
Immunostaining CA 125					
Positive	28	16	2	13	1
Negative	2	1	2	1	4
Immunostaining, CEA					
Positive	7	8	1	6	5
Negative	23	9	3	8	0
Serum, CA 125	36	19	5	17	8
> 35 u/ml	19	6	2	13	2
0 - 35 u/ml	17	13	3	4	6

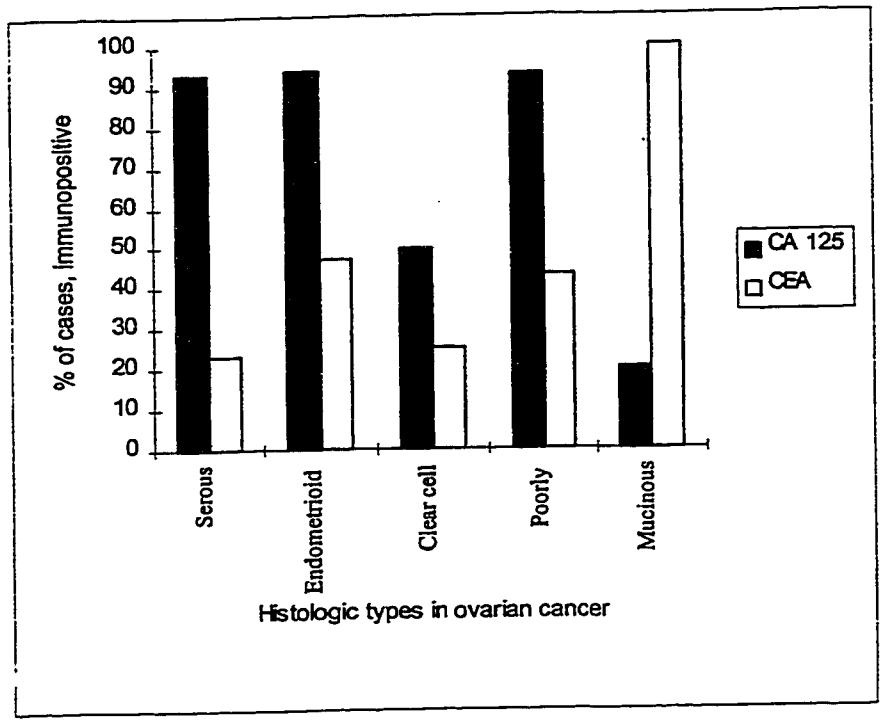

Figure 9: CA125 and CEA immunopositive cases in patients tumor, PET, various histologic types of EOC cases.

There was statistically significant difference in patient median survival and RFS between normal and elevated serum CA 125 levels ($p<0.001$, and $p<0.001$ respectively), shown in Figure 10 and 11 and Table 10. Table 11 shows CA 125 serum levels and the histologic origin of tumors in the study. The most common type of malignancy was serous type (n = 36, 41%), followed by undifferentiated type (n = 16, 18%), endometrioid type (n = 19, 22%), mucinous type (n = 7, 8%), clear cell type (n = 5, 6%), and data on four cases was missing.

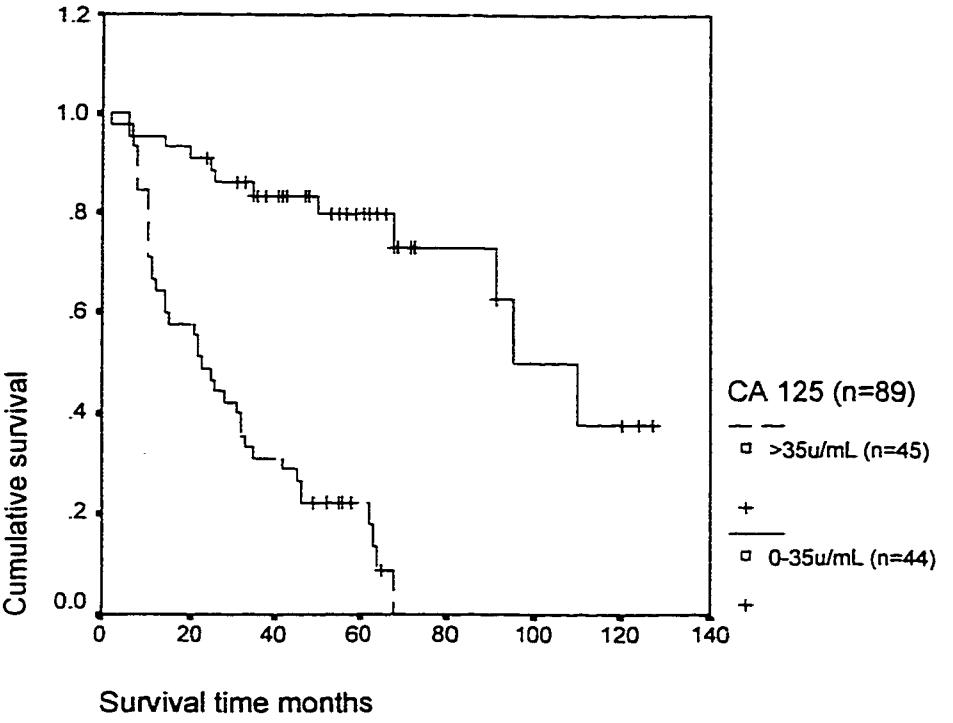

Figure 10: Survival analysis for ovarian cancer patients with CA125 level measurements, 0-35 u/ml, median months, 110 , >35 u/ml, median months, 23, $p <$ *0.001.*

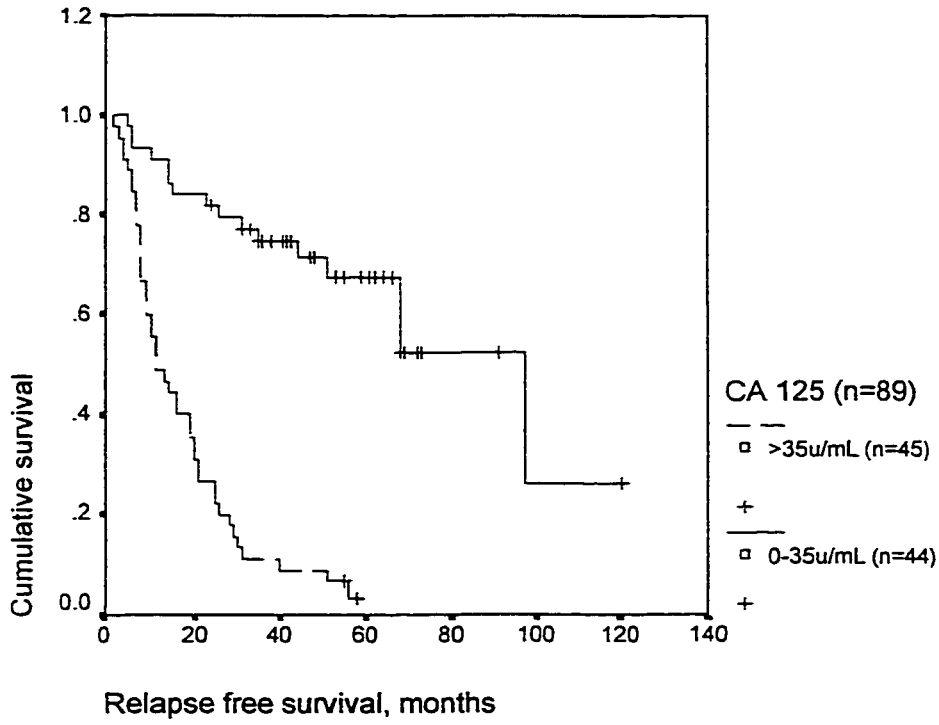

Figure 11: Analysis for ovarian cancer patients in relapse-free survival with CA125 level measurements, 0-35 u/ml, median months, 97, >35 u/ml, median months, 11, *p* < *0.001.*

In this retrospective study CEA tissue overexpression was observed in all mucinous ovarian carcinoma cases (Table 12).

Table 12: Mucinous carcinoma cases and the relationship to serum CA 125, CA 125 immunostaining and CEA immunostaining. CA 125 serum level: 1, 0-35 u/ml and 2, > 35 u/ml. CA 125 immunostaining, and CEA immunostaining: 0, negative, and 1, positive.

Case	CA 125 serum level	CA 125 immunoreactivity	CEA Immunoreactivity
1	1	0	1
2	2	No data	No data
3	1	0	1
4	1	0	1
5	1	No data	No data
6	2	0	1
7	1	1	1

4.2 Immunohistochemical detection of protein expression Erb-B2, $P16^{INK4A}$, $P21^{WAF\backslash CIP1}$, P53, and MSH2 in patients with ovarian cancer

4.2.1 Immunohistochemical detection of ErbB-2 overexpression

In Figure 12 ErbB-2 overexpression can be seen as demonstrated by membrane staining. In the current study of ErbB-2 protein expression in EOC patients, there were 32 (30%) cases with increased expression and the remaining 75 (70%) cases were negative. Overexpression of ErbB-2 protein did not have an adverse impact on survival and RFS, $p = 0.5$, and $p = 0.2$ respectively as reported in Table 13.

Figure 12: Immunohistochemical membrane staining of a p185-positive (ErbB-2) endometrioid ovarian carcinoma.

Table 13: Immunoreactivity of ErbB-2, $p16^{INK4A}$, $p21^{WAF1/CIP1}$, p53, and MSH2 in PET and survival analysis of ovarian cancer patients considered.

Immunoreactivity score	Cases(%)	Survival, months (median)	P value	RFS, months (median)	P value
ErbB-2			0.5		0.22
0	75(70)	62		35	
1	32(30)	64		83*	
$P16^{INK4A}$			0.57		0.58
0	61(57)	63		56	
1	46(33)	106		58	
$P21^{WAF1/CIP1}$			0.74		0.97
0	63(59)	64		56	
1	44(31)	52		78*	
P53		Ψ^1	0.04	Ψ^2	0.008
0	68(61)	92*		88*	
1	44(39)	48		28	
MSH2			0.77		0.77
0	37(34)	106		77	
1	70(66)	64		40	

0: Negative, 1: Positive, RFS: Relapse free survival, *: Mean survival, Ψ^1: Relative risk of death: 4.99 (95% Confidence interval: 0.79-31.46) and Ψ^2: relative risk of relapse: 1.99 (95% Confidence interval: 1.17-3.37).

4.2.2 Immunohistochemical detection $P16^{INK4A}$ overexpression

Representative example of $P16^{INK4A}$ protein accumulation in the ovarian cancer cells is shown in Fig. 13. $P16^{INK4A}$ overexpression in 46 cases (33%) did not have adverse effect on survival and RFS in ovarian cancer patients as shown in Table 13.

Figure 13: Immunohistochemical staining of $p16^{INK4A}$ protein from a paraffin section show, nuclear positive tumor cells.

4.2.3 Immunohistochemical detection of $P21^{WAF1/CIP1}$ expression

An example of $P21^{WAF1/CIP1}$ immunopositive case with endometrioid carcinoma in our study as shown in Figure 14. Of the 107 cases, 44 (41%) tumors were $P21^{WAF1/CIP1}$ positive and 63 (59%) were $P21^{WAF1/CIP1}$ negative. The results of survival analysis demonstrated no difference in overall survival and RFS between patients with $P21^{WAF1/CIP1}$ expression tumors and those with $P21^{WAF1/CIP1}$ negative tumors are reported in Table 13.

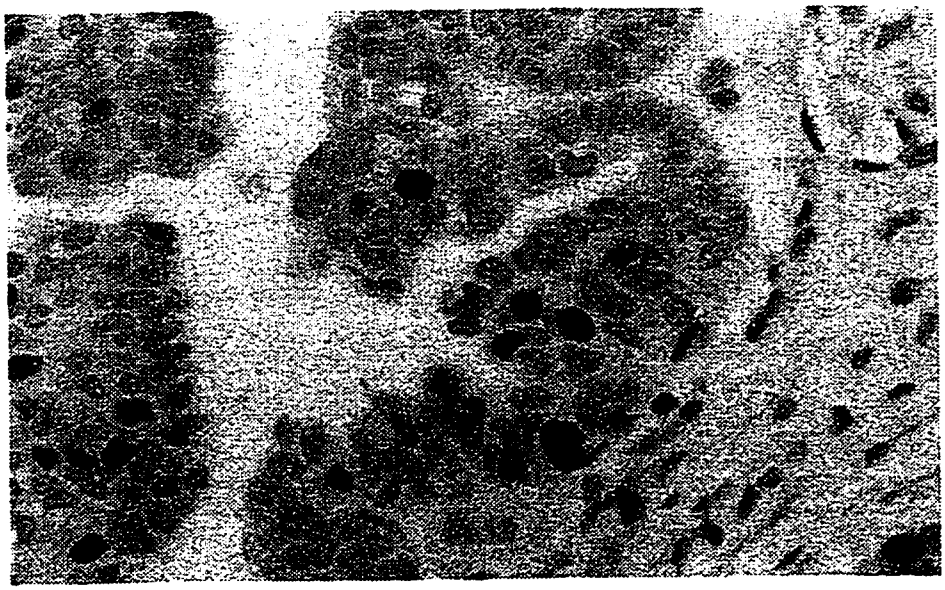

Figure 14: Immunohistochemical staining for the P21WAF1/CIP1 protein of an endometrioid ovarian carcinoma.

The results of the immunoreactivity of $P21^{WAF1/CIP1}$ protein and association with P53 expression are summarized in Table 14. $P21^{WAF1/CIP1}$ expression was associated with P53 overexpression in 11 cases (27%) as shown in Table 14. In cases with P53 overexpression (40) out of 107, there was statistically significant relationship to decreased survival among $P21^{WAF1/CIP1}$ expressors. The patients were grouped within P53 overexpressors according to $P21^{WAF1/CIP1}$ expression to determine the influence on survival. The cases with $P21^{WAF1/CIP1}$ expression, 11 cases (27%), had a shorter overall median survival, 29 median survival months, than the patients with negative $P21^{WAF1/CIP1}$ staining, 62 median months of survival, $p = 0.03$.

Table 14: Association between $p21^{WAF1/CIP1}$ and p53 expression in ovarian cancer.

P53	**$P21^{WAF1/CIP1}$**		**P value**
	Negative n(%)	**Positive n(%)**	
Negative	34(51)	33(49)	0.04
Positive	29(73)	11(27)	

4.2.4 Immunohistochemical detection of P53 overexpression

P53 overexpression in a case with clear cell carcinoma in our study is shown in Fig. 15. The accumulation of mutated P53 protein detected in the nucleus of PET was related to shorter survival and RFS in 44 cases (39%) with EOC (Table 13) as shown in Fig. 16.

Forty four cases (39%) showed P53 overexpression. There was a statistical significant association between P53 overexpression and $P16^{INK4A}$ immunonegative staining in 18 cases (29%) out of 61 cases, $p = 0.05$ as shown in Table 15. The overexpression of P53 protein in the $P16^{INK4A}$ immunonegative cases was not found to be related to survival, $p = 0.4$.

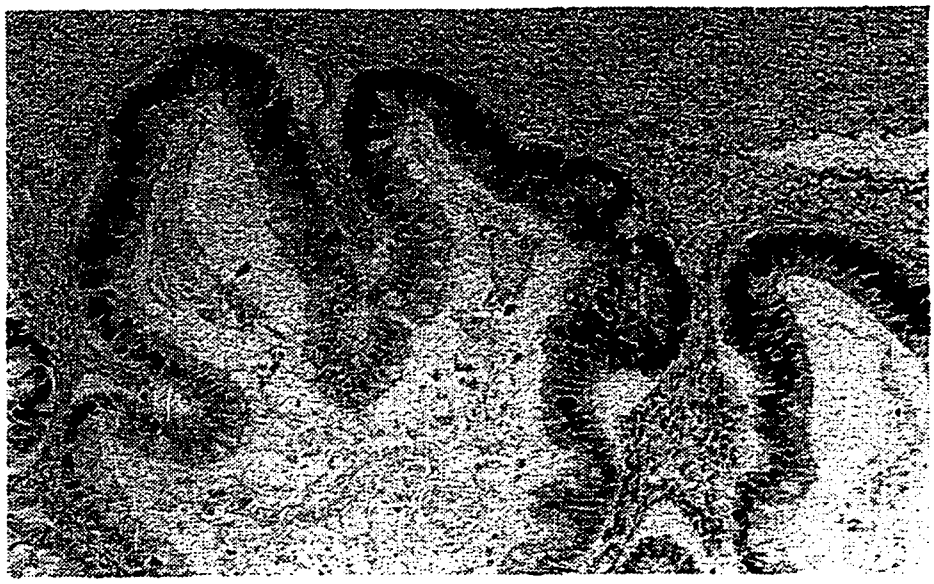

Figure 15: P53 protein accumulation in the nuclei of a patient with clear cell carcinoma of the ovary.

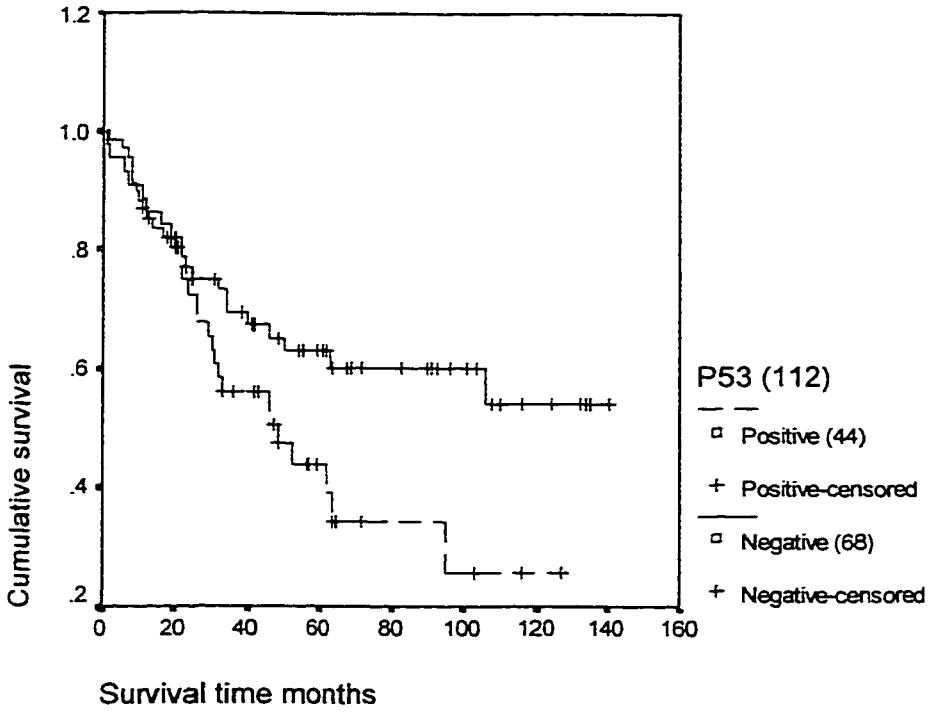

Figure 16: Survival analysis of p53 overexpression in 44 cases (41%) association with decreased survival compared to P53 negative 68 cases (59%), $p = 0.04$.

Table 15: Association between expression of p53 and $p16^{INK4A}$ in ovarian cancer.

	P53		
$P16^{INK4A}$	Negative n(%)	Positive n(%)	P value
Negative	43(71)	18(29)	0.05
Positive	24(52)	22(48)	

4.2.5 Immunohistochemical detection of MSH2 expression

Fig. 17 illustrates expression of MSH2 protein in a case with serous adenocarcinoma in this study. The MSH2 protein expression was detected in 70 cases (66%) and the remaining 37 cases (34%) were negative for MSH2 protein (Table 13). There was no statistically significant difference in prognosis between the MSH2 positive and negative tumors in this study. Although, there was decreased survival and RFS in the patients with MSH2 inactivation by immunohistochemical detection of the protein.

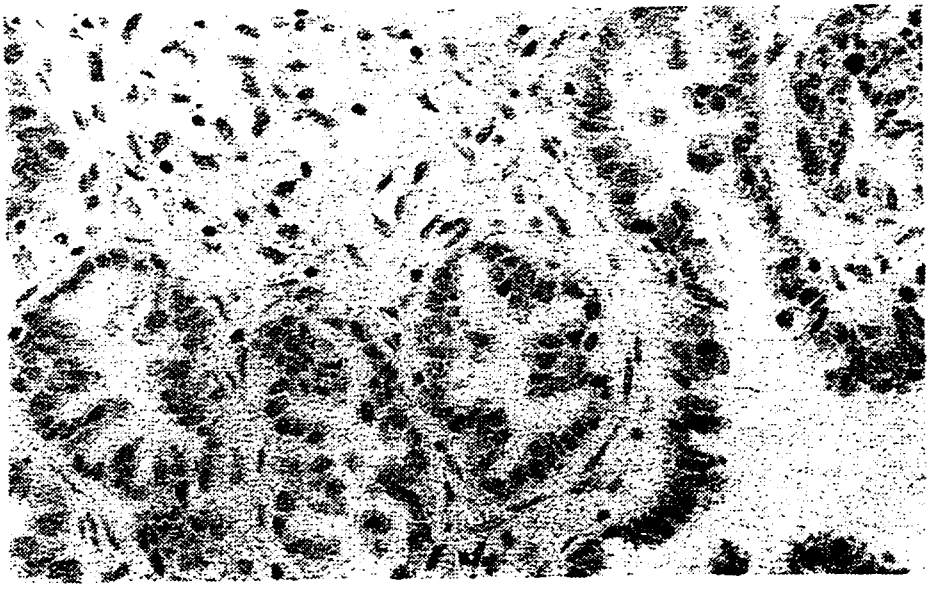

Figure 17: Positive nuclear immunoreactivity of MSH2 protein in serous cystadenocarcinoma patient.

4.3 Dot blot hybridization to identify genetic alterations in DNA from archival paraffin embedded tissue specimens

A representative autoradiogram (Figure 18) of dot blot hybridization shows the different types of genetic changes (deletions and/ amplification) in the DNA extracted from the PET blocks of primary ovarian cancer cases. Of the sixty-three cases of primary ovarian carcinoma there were genetic changes in at least 59 cases (94%).

The results obtained included deletions ($p16^{INK4A}$ = 22 cases, RB1 = 19 cases, CDK4 = 28 cases, Cyclin D1 = 23 cases), and amplification ($p16^{INK4A}$ = 7 cases, RB1 = 19 cases, CDK4 = 7 cases, Cyclin D1 = 10 case) (Table 16). Amplification and deletion changes in the genes were evaluated by dot blot hybridization to identify a subset of patients with differences in overall survival and RFS (Table 16). Furthermore, data in Table 17 shows no differences in adverse outcome events were identified for the alterations, such as deletion and amplification, in the genes examined.

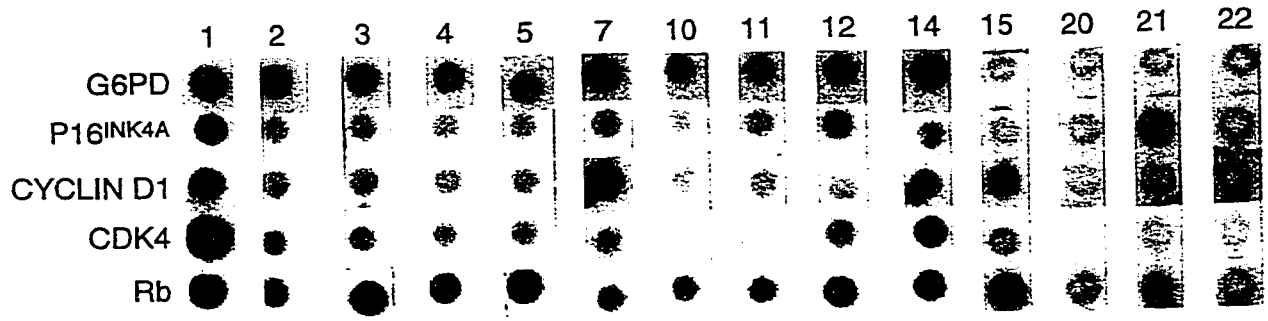

Figure 18: Representative autoradiogram of 10μg of genomic DNA extracted from archival paraffin embedded material, blotted onto a membrane and hybridized to a sequence complementary to $p16^{INK4A}$ and a diploid control sequence, G-6-PD. Representative cases of deletion, possible deletion and amplification in the above autoradiogram are: Case 1 showed only amplification of CDK4, Cases 2, 4, 10, 11(showed deletion{significant reduction in signal, less than 50%} of $p16^{INK4A}$, CDK4 and Cyclin D1, case 7 showed deletion of $p16^{INK4A}$ and CDK4, Possible deletion(showed reduction in signal, less than 25%) of RB1 as well as amplification of Cyclin D1, Case 14 showed deletion of $p16^{INK4A}$ and Case 21 showed 2-fold amplification of $P16^{INK4A}$ and RB1 and in addition deletion of Cyclin D1.

Table 16: Survival analysis of genes considered for deletion/amplification by dot blot hybridization.

Gene	Total n = 63 (%)	5 Year survival rate	P value	5 Year RFS rate	P value
$P16^{INK4A}$					
Deletion			0.8		0.9
Normal*	28 (44)	44		51	
Deleted	28 (44)	58		55	
Amplification			0.4		0.9
Normal*	28(44)	44		51	
Amplified	7 (12)	75		43	
Cyclin D1					
Deletion			0.9		0.8
Normal*	30 (48)	54		54	
Deleted	23 (37)	58		58	
Amplification			0.4		0.1
Normal*	30(48)	55		58	
Amplified	10 (15)	45		26	
CDK4					
Deletion			0.3		0.3
Normal*	28 (44)	62		62	
Deleted	28 (44)	47		43	
Amplification			0.8		0.4
Normal*	28(44)	62		64	
Amplified	7 (12)	54		42	
Rb-1					
Deletion			0.4		0.6
Normal*	25 (40)	43		43	
Deleted	19 (30)	58		52	
Amplification			0.1		0.1
Normal*	25(40)	43		43	
Amplified	19 (30)	65		64	

*Normal: Neither amplification or deletion when compared to internal control signal, RFS: Relapse free survival.

Table 17: Association of gene deletion/amplification by dot blot hybridization with adverse outcome.

Gene	Alive, at 5 years Total n = 39 (%)	Died within 5 years Total n = 24 (%)	P value
$P16^{INK4A}$			
Deletion			0.6
Normal*	15 (54)	13 (46)	
Deleted	18 (64)	10 (36)	
Amplification			0.2
Normal*	15 (54)	13 (46)	
Amplified	6 (86)	1 (14)	
Cyclin D1			
Deletion			0.8
Normal*	18 (60)	12 (40)	
Deleted	15 (65)	8 (35)	
Amplification			1
Normal*	18 (60)	12 (40)	
Amplified	6 (60)	4 (40)	
CDK4			
Deletion			0.6
Normal*	19 (68)	9 (32)	
Deleted	16 (57)	12 (43)	
Amplification			0.7
Normal*	19 (68)	9 (32)	
Amplified	4 (57)	3 (43)	
Rb-1			
Deletion			0.5
Normal*	13 (60)	12 (40)	
Deleted	12 (63)	7 (37)	
Amplification			0.2
Normal*	13 (60)	12 (40)	
Amplified	14 (74)	5 (26)	

*Normal: Neither amplification or deletion when compared to internal control signal.

Gene alterations evaluated by dot blot hybridization was examined in conjunction with DNA content data as shown in Table 18. In the cases with no change Cyclin D1 by dot blot hybridization, the proportion of diploid cases was significantly higher than the

proportion of aneuploid cases, $p = 0.07$ (Table 18). RB1 gene was found to be diploid in 13 (93%) cases out of 14 cases with deleted RB1 gene by dot blot hybridization, $p = 0.05$. The alterations in $p16^{INK4A}$ and CDK4 genes were not associated with DNA content.

Table 18: Association of gene deletion/amplification by dot blot hybridization with DNA content.

Gene	Aneuploid Total n = 13 (%)	Diploid Total n = 37 (%)	P value
$P16^{INK4A}$			
Deletion			1.0
Normal*	6 (26)	17 (74)	
Deleted	6 (37)	16 (73)	
Amplification			1.0
Normal*	6 (26)	17 (74)	
Amplified	1 (20)	4 (80)	
Cyclin D1			
Deletion			0.3
Normal*	4 (15)	22 (85)	
Deleted	5 (31)	11 (69)	
Amplification			0.07
Normal*	4 (15)	22 (85)	
Amplified	4 (50)	4 (50)	
CDK4			
Deletion			1.0
Normal*	6 (26)	17 (74)	
Deleted	5 (24)	16 (76)	
Amplification			1.0
Normal*	6 (26)	17 (74)	
Amplified	2 (33)	4 (67)	
Rb-1			
Deletion			0.05
Normal*	8 (40)	12 (60)	
Deleted	1 (7)	13 (93)	
Amplification			0.5
Normal*	8 (40)	12 (60)	
Amplified	4 (25)	12 (75)	

*Normal: Neither amplification or deletion when compared to internal control signal.

The p53 antibody was found to stain positively in 18 cases (33%) and negative in 36 cases (67%). A correlation between p53 immunoreactivity and alterations in $p16^{INK4A}$, RB1, CDK4 and Cyclin D1 indicates these genes were altered, however not statistically significant (Table 19). Three of the four cases with no genetic changes in the genes examined had p53 overexpression.

Table 19: Association of gene deletion/amplification by dot blot hybridization with p53 immunoreactivity.

Gene	P53 immunoreactivity		P value
	Negative n=36(%)	Positive n=18(%)	
$P16^{INK4A}$			
Deletion			0.4
Normal*	14 (58)	10 (42)	
Deleted	18 (72)	7 (28)	
Amplification			0.6
Normal*	14 (58)	10 (42)	
Amplified	4 (80)	1 (20)	
Cyclin D1			
Deletion			1.0
Normal*	19 (70)	8 (30)	
Deleted	12 (67)	6 (33)	
Amplification			0.4
Normal*	19 (70)	8 (30)	
Amplified	5 (56)	4 (44)	
CDK4			
Deletion			1.0
Normal*	16 (67)	8 (33)	
Deleted	16 (69)	7 (31)	
Amplification			0.7
Normal*	16 (67)	8 (33)	
Amplified	4 (57)	3 (43)	
Rb-1			
Deletion			0.5
Normal*	12 (57)	9 (43)	
Deleted	11 (69)	5 (31)	
Amplification			0.3
Normal*	12 (57)	9 (43)	
Amplified	13 (76)	4 (24)	

*Normal: Neither amplification or deletion when compared to internal control signal.

4.4 Immunohistochemical detection of P53 protein expression, relationship with DNA content and tumor factors

Data in Table 20 shows there was a highly significant difference in overall survival and RFS when patients with P53 immunonegative tumors were grouped into diploid and aneuploid DNA content categories $p = 0.01$, and $p = 0.005$ respectively. The patients with >5.0% S content in the immunonegative patients had a significantly shorter survival and RFS than the patients with <5.0% S content, $p = 0.01$, and $p = 0.01$ respectively (Figure 19 and 20).

Table 20: P53 immunoreactivity and association with DNA content and % S content in ovarian cancer cases.

Factor	Total n=68(%)	5 Year survival rate	RR of death	95% CI	P value	5 Year RFS rate	RR of relapse	95% CI	P value
P53 Immunonegative Ploidy status					0.01				0.005
Diploid	53 (78)	76	1	-		72	1	-	
Aneuploid	15 (22)	28	5.4	0.33-86.52		25	2.95	1.33-6.53	
% S content					0.01				0.01
</= 5.0	47 (69)	78	1	-		75	1	-	
> 5.0	21 (31)	35	3.28	0.19-55.80		35	2.60	1.20-5.63	
P53 Immunopositive Ploidy status					0.99				0.93
Diploid	25(57)	36	*	-		32	1	-	
Aneuploid	19(43)	32	*			38	1.03	0.50-2.14	
% S content									
</= 5.0	18(41)	78	*	-	0.01	75	1	-	0.01
> 5.0	26(59)	35	*			35	1.29	0.62-2.70	

RR: relative risk, CI: confidence interval, RFS: Relapse free survival, * reliable estimates could not be obtained due to few failure events.

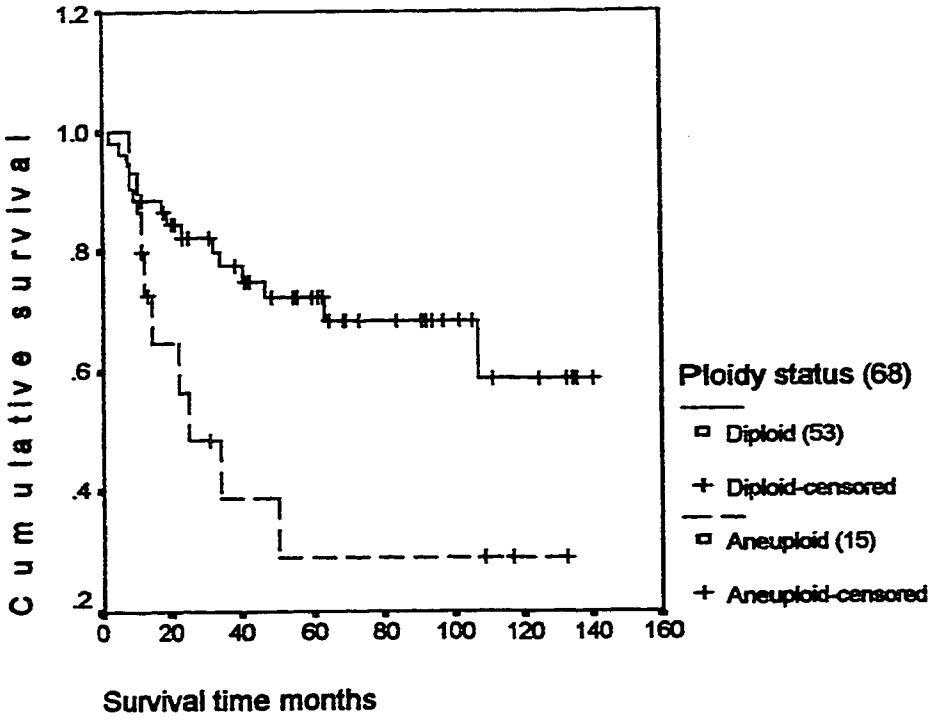

Figure 19: Survival rates within p53 negative tumors and diploid DNS content (53 cases, 47%) were significantly different from the aneuploid tumors (15 cases, 13%), *p=0.01*.

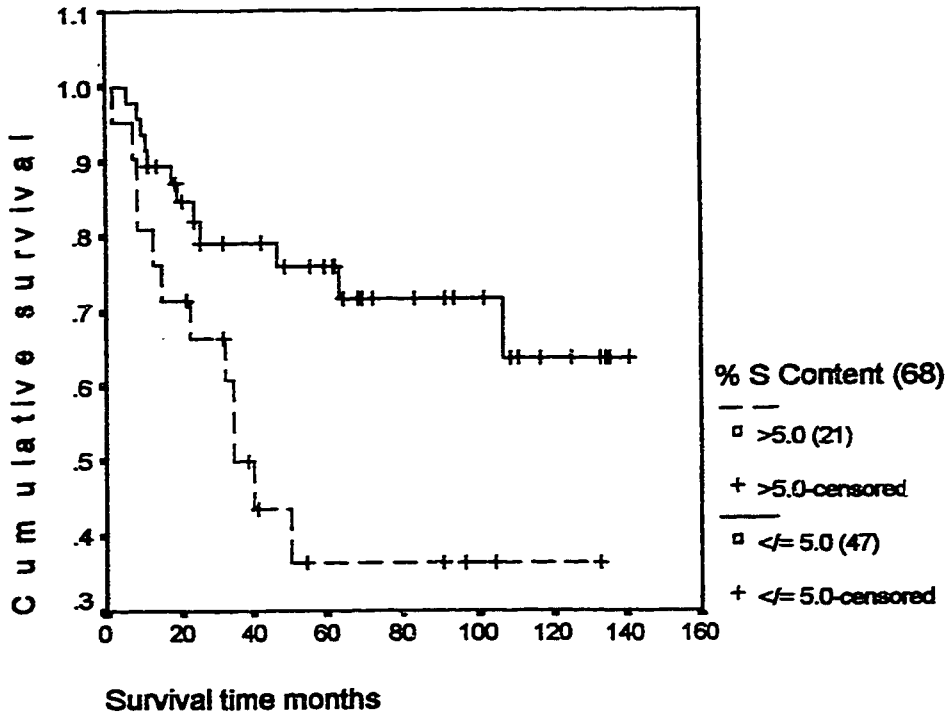

Figure 20: The survival rate for patients with p53 negative tumors and > 5.0% S content was significantly lower (21 cases, 31%) than for patients with <5.0% S content (47 cases, 69%), *p = 0.01.*

Tumor factor such as stage, and some tumor specific factors like grade, and residual disease were examined for their relationship with P53 immunoreactivity and outcome events. From the data in Table 21, it can be seen that patients with grade 2 ovarian cancer, and P53 positive tumors, 11(35%) out of 31 had a significantly worse survival, and RFS than the patients with P53 negative staining ($p = 0.05$, and $p = 0.01$). FIGO stage, grade 1 and 3, and residual disease were not found to be associated with prognosis in ovarian cancer patients. Figure 21, 22, and 23 represent survival curves of P53 immunoreactivity within grade 2, NED, and residual disease cases.

Table 21: Patient survival rates according to tumor factors and p53 overexpression.

Disease parameters	Total n=112(%)	5 Year survival rate	P value	5 Year RFS rate	P value
Early stage			0.6		0.1
P53 negative	47 (42)	80		77	
Positive	17 (15)	78		62	
Advanced stage			0.9		0.9
P53 negative	19 (17)	22		20	
Positive	25 (22)	18		18	
Missing	4 (4)				
Grade 1			0.7		0.9
P53 negative	19 (17)	83		73	
Positive	8 (7)	65		70	
Grade 2		Ψ[1]	0.05	Ψ[2]	0.01
P53 negative	20 (18)	83		85	
Positive	11 (10)	45		33	
Grade 3			0.6		0.7
P53 negative	25 (22)	33		25	
Positive	23 (21)	42		22	
Missing	6 (5)				
NED			0.6		0.3
P53 negative	36 (33)	83		82	
Positive	18 (16)	65		68	
Residual disease			0.7		0.6
P53 negative	25 (22)	22		21	
Positive	24 (21)	20		17	
Missing	9(8)				

Ψ[1]: Reliable estimates could not be obtained due to few failure events, Ψ[2]: relative risk of relapse: 4.78 (95% Confidence interval: 1.22-18.61).

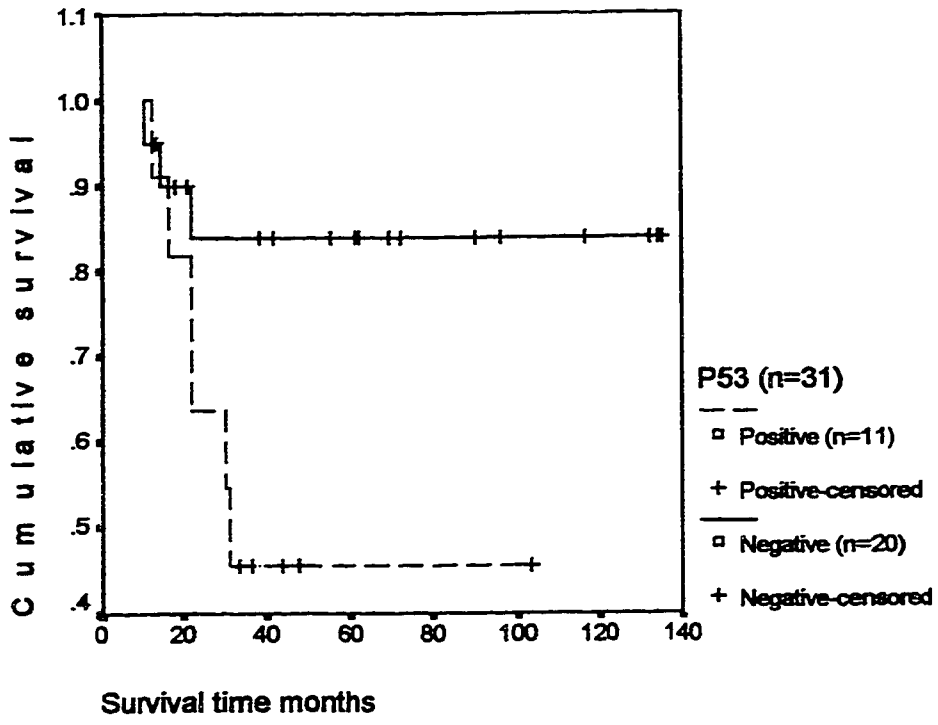

Figure 21: Survival analysis in grade 2 EOC cases, and their relationship with p53 protein expression, *p= 0.05*.

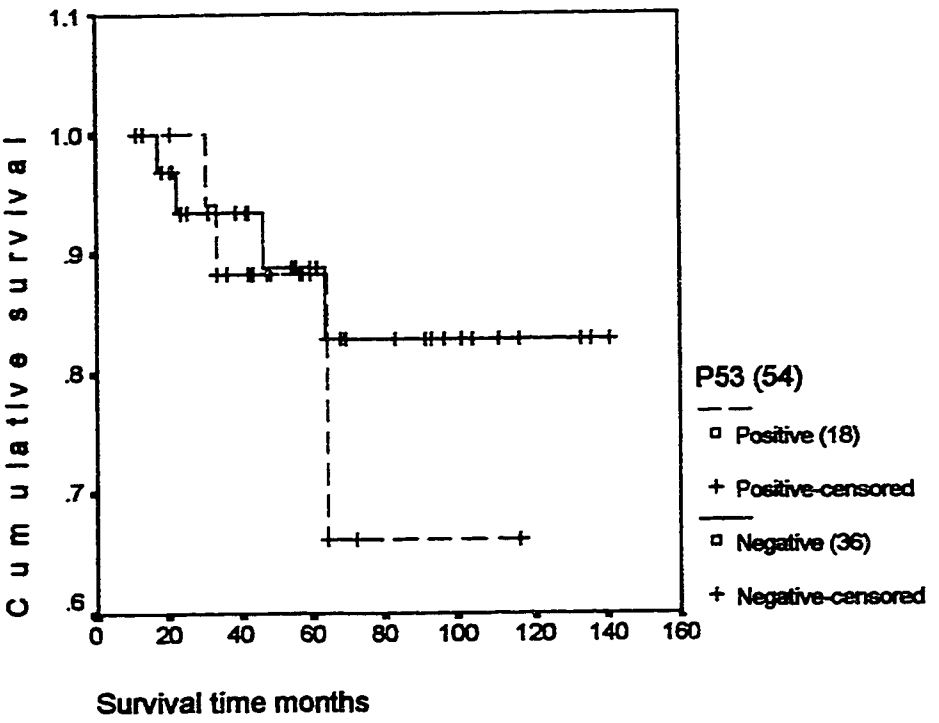

Figure 22: Survival analysis of EOC cases with NED and p53 expression, $p = 0.6$, not associated with overall survival.

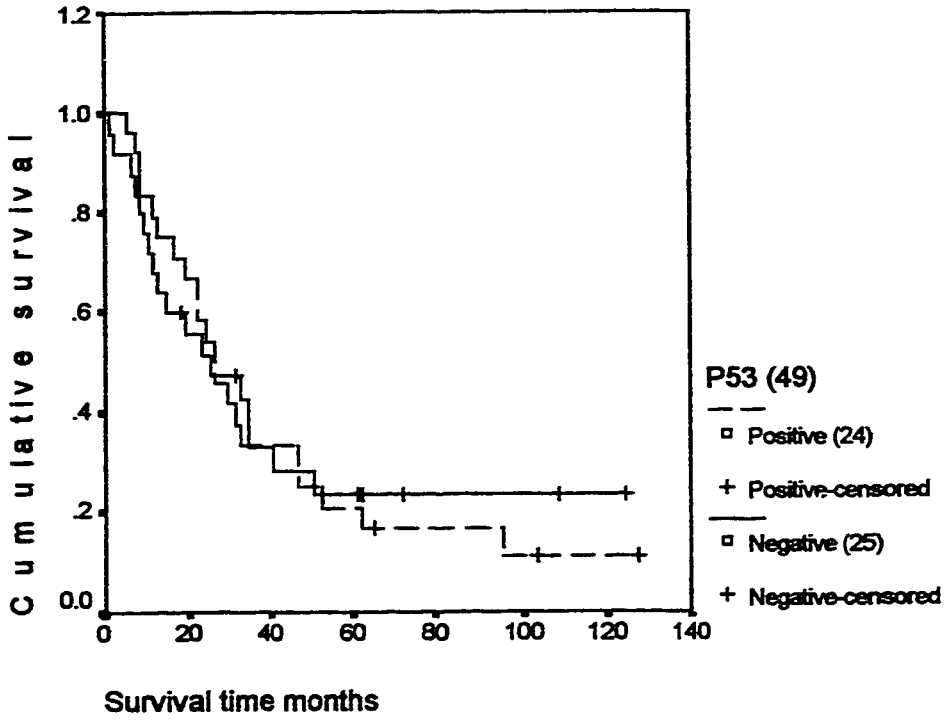

Figure 23: Survival analysis of EOC cases with residual disease and p53 protein expression no association with overall survival, $p = 0.7$.

Abnormal DNA content, proliferative rate, and P53 immunoreactivity have a significant influence on adverse outcome in ovarian cancer patients. The effect of % S content was the most significant factor affecting survival at the end of 5 years, $p = 0.001$, followed by DNA content, $p = 0.01$, and P53 expression, $p = 0.02$ (Table 22).

Table 22: The relationship of DNA content, % S content and p53 immunoreactivity and an adverse outcome.

Flow cytometry	Alive at 5 years Total n=64(%)	Died within 5 years Total n=48(%)	P values
DNA content			
Aneuploid	13(38)	21(62)	0.01
Diploid	51(65)	27(35)	
% S Content			
</=5.0	46(71)	19(39)	0.001
>5.0	18(38)	29(62)	
P53			
Negative	45(66)	23(34)	0.02
Positive	19(43)	25(57)	

Table 23, and Figure 24 show that the survival rates were significantly low (44%) for P53 positive, and aneuploid patients than P53 negative and diploid tumors (72%), $p = 0.02$. In addition, we also found P53 negative tumors with diploid content when categorized into < 5.0 %, and > 5.0% S content, the former group of patients had a much better prognosis (82%, 5 year survival rate) than the latter group (45%, 5 year survival rate), Figure 25, and Table 23.

Table 23: Survival rates of patients grouped according to p53 overexpression, DNA content and % S content.

Factors, Flow cytometry P53	Total	5 Year survival rate	P value
	n = 72 (%)		
Negative and diploid	53 (74)	72	0.02
Positive and aneuploid	19 (26)	44	
Negative and diploid			
% S content	n=53(%)		0.01
</= 5.0	39 (73)	82	
> 5.0	14 (27)	45	

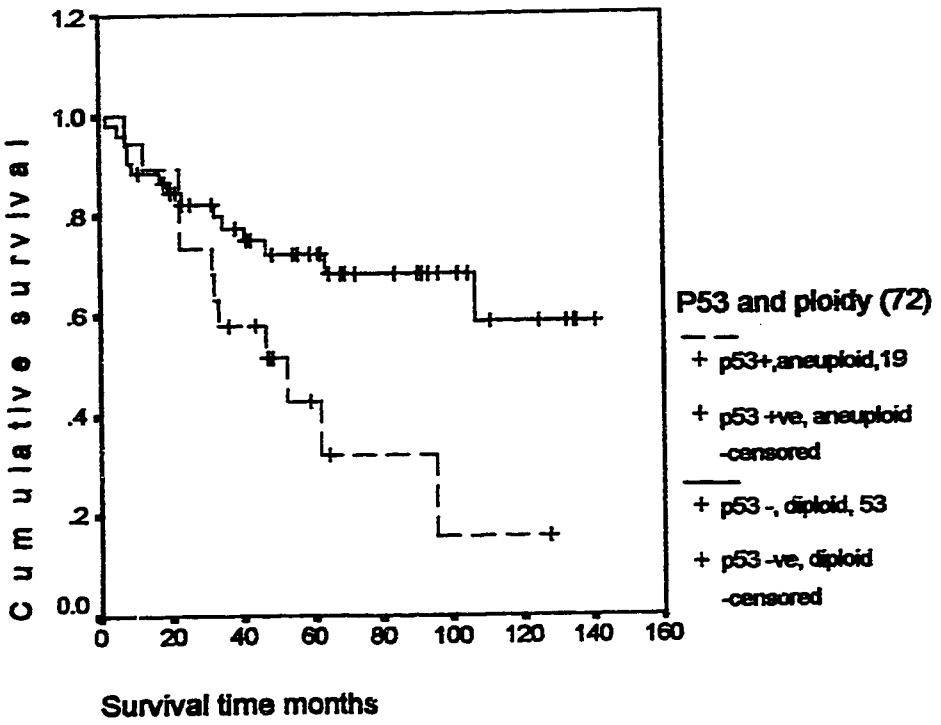

Figure 24: Survival analysis when patients were grouped by P53 negative, and diploid DNA cases compared to P53 positive, and aneuploid tumors, a statistically significant difference was noticeable, $p = 0.02$.

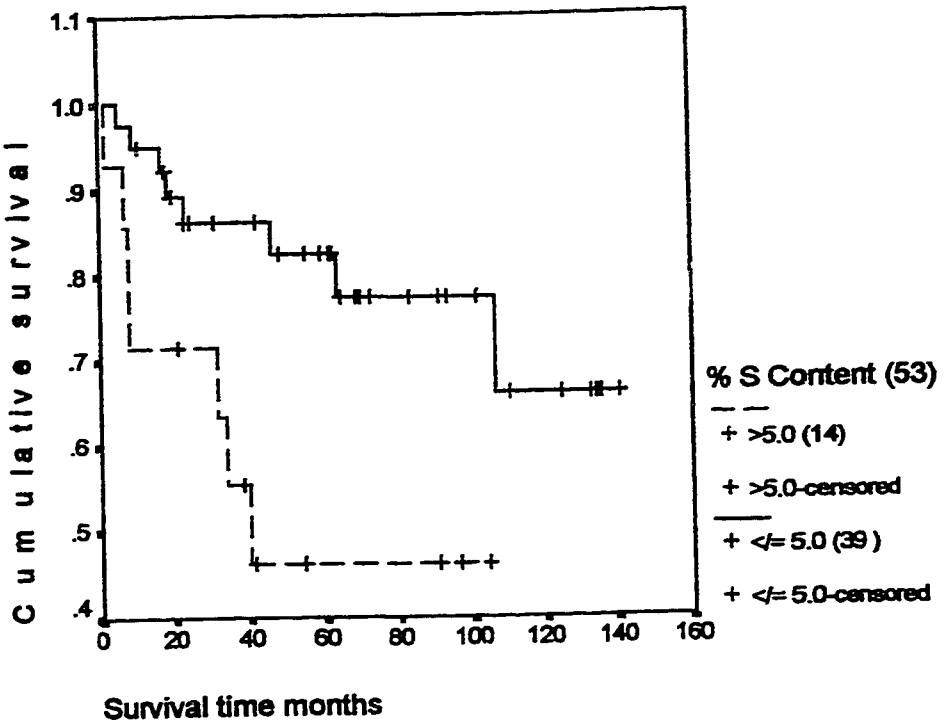

Figure 25: Consideration of all P53 negative and diploid cases when grouped into <5.0% S content, and >5.0% S content, a significantly different survival rate in the latter group was noted, *p = 0.01*.

4.5 Tumor ploidy and S phase content evaluated in PET from ovarian cancer patients

Typical DNA histograms representing diploid and aneuploid DNA content from PET from ovarian cancer patients are shown in Figure 26 a and b respectively.

Figure a.

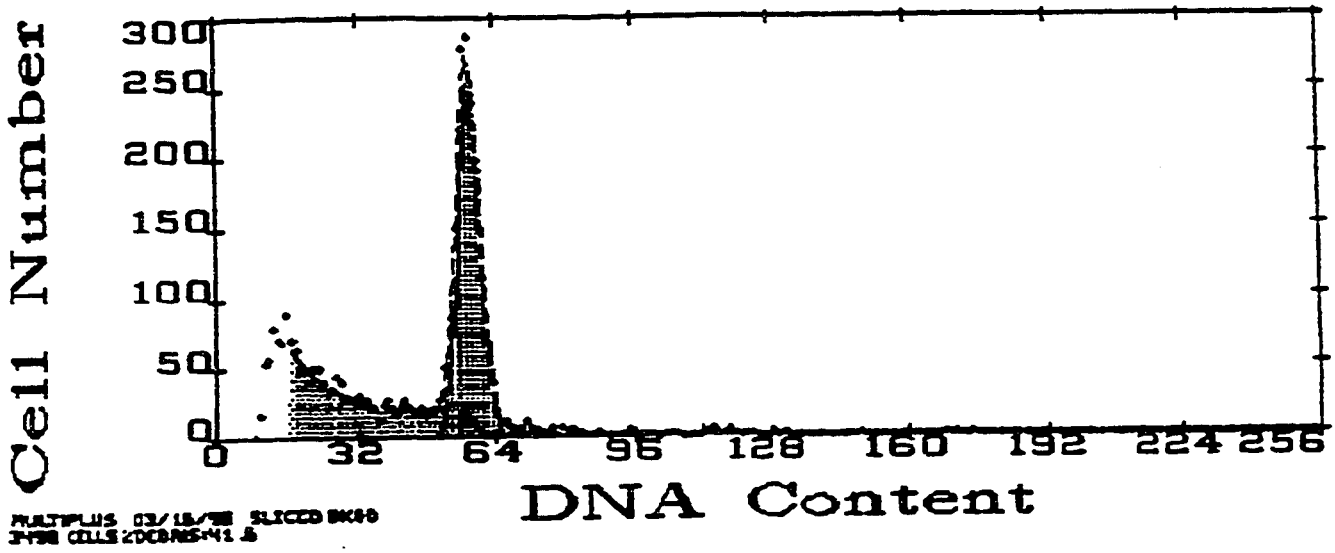

Figure b.

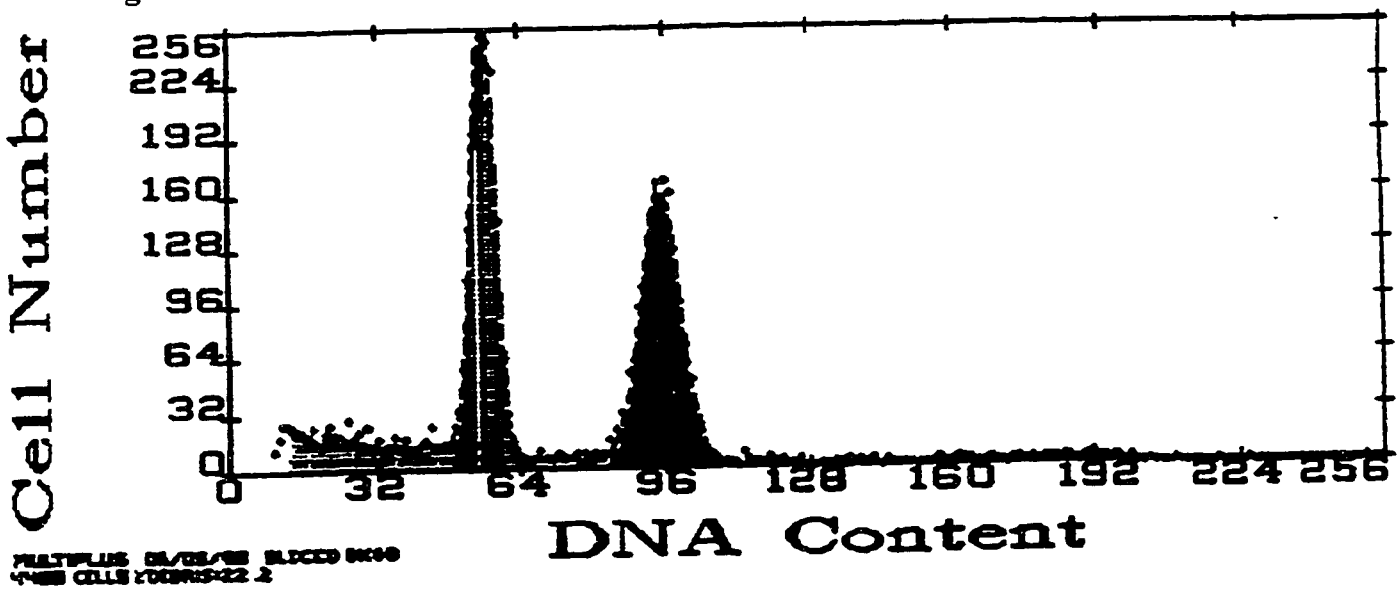

Figure 26: Illustration of DNA histograms obtained from flow cytometric analysis of PET from ovarian cancer patients. a: diploid, and b: aneuploid. On the x-axis, channel number represents relative fluorescence intensity, which is directly proportional to DNA content and number of cells are shown on the y-axis.

In the following Table 24 results of DNA content and % S content relationship with survival and RFS can be seen. Overall there were 112 patients whose data was evaluable in this study. The histograms on being classified into diploid and aneuploid categories resulted in 78 cases (70%) and 34 cases (30%) on the two groups respectively. The 5 year survival rates in the aneuploid cases was significantly shorter, 38%, than the diploid cases, 64% $p = 0.03$ (Figure 27). Using DNA content as a prognostic marker of RFS, 78 patients (70%) with diploid DNA content were found to have a better prognosis than the patients who had not shown abnormal DNA content, $p = 0.01$ (Figure 28).

Subsequently, we investigated the % S content obtained from cell cycle analysis in each case. Table 24 shows the data obtained as for % S content on grouping according to median % S content, a significant proportion of cases were with <5.0% S content, 58% (65 cases) and the remaining 47% (42 cases) were in the > 5.0% S content category. The survival analysis indicated that % S content was significantly associated with survival and RFS, $p = 0.0008$, and $p = 0.004$ respectively (Figure 29, and 30).

Table 24: Survival analysis of DNA content and % S content in patients.

Flow cytometry	Total n=112(%)	5 Year survival rate	P value	5 Year RFS rate	P value
DNA content			0.03		0.02
Aneuploid	34 (30)	38		36	
Diploid	78 (70)	64		57	
% S content			0.0008		0.004
</= 5.0	65 (58)	70		58	
> 5.0	47 (42)	32		37	

Furthermore, we analyzed the DNA content and % S content along with tumor factor. We found that both DNA content and % S content were significantly associated with stage (Table 25), however, % S content was more significantly associated with stage than DNA content in this investigation.

Table 25: DNA content and % S content association with FIGO stage in patients.

Flow cytometry	Early Stage Total n = 64 (%)	Advanced Stage Total n = 44 (%)	P value
DNA content			0.02
Aneuploid	13 (41)	19 (59)	
Diploid	51 (67)	25 (33)	
% S content			<0.001
</= 5.0	47 (73)	17 (27)	
> 5.0	17 (38)	27 (62)	

Table 26 depicts the prognosis of the tumor factors of the patients when grouped by DNA content. Out of the 64 patients in ES there were at least 51 cases (46%) without abnormality in DNA content as detected by flow cytometry and these cases had better prognosis, 5 year RFS rate, 74%, than the cases with aneuploid cell populations, $p = 0.05$ as shown in Table 26. However, aneuploidy was not found to be related to overall survival in ES and AS patients.

In patients with grade 2 cancer the effect of DNA content was significantly associated with survival and RFS in Figure 31 and Table 26. Survival analysis in patients with grade 1 and grade 3 cancers, and aneuploid DNA content was not a significant factor associated with outcome in ovarian cancer patients. Furthermore, the histologic type of the tumor as recorded from the medical charts, and DNA content by flow cytometry were

analyzed to determine their association with prognosis in these patients. The data indicated that among the serous histologic type there was a trend toward significantly adverse effect on survival and RFS in patients with aneuploid DNA content as shown in Table 26. It was also interesting to note that the mucinous carcinomas were almost exclusively diploid in all the cases. The results reported in Table 26 shows that there was only one mucinous tumor case with abnormal DNA content and prognosis was significantly poor than the diploid cases. Residual disease and tumor size at laparotomy were apparently not found to be associated with DNA content as shown in Table 26.

Table 26: Survival analysis, tumor factors with DNA content in ovarian cancer patients.

Tumor factor	Total n=112(%)	5 Year survival rate	P value	5 Year RFS rate	P value
Early Stage			0.6		0.05
Aneuploid	13 (12)	72		58	
Diploid	51 (46)	85		74	
Advanced stage			0.6		0.9
Aneuploid	19 (17)	18		17	
Diploid	25 (22)	25		19	
Missing	4 (3)				
Grade 1			0.62		0.6
Aneuploid	3 (3)	66		73	
Diploid	24 (21)	80		106	
Grade 2			0.007		0.002
Aneuploid	13 (12)	42		35	
Diploid	18 (16)	88		89	
Grade 3			0.8		0.6
Aneuploid	18 (16)	34		22	
Diploid	30 (27)	42		25	
Missing	6 (5)				
NED			0.6		0.1
Aneuploid	13 (12)	82		64	
Diploid	41 (36)	92		81	
Residual disease			0.9		0.9
Aneuploid	20 (18)	20		26	
Diploid	29 (26)	22		18	
Missing	9 (8)				
Histologic type					
Serous			0.06		0.07
Aneuploid	21 (19)	32		32	
Diploid	27 (24)	68		60	
Undifferentiated			1		0.9
Aneuploid	5 (4)	40		20	
Diploid	12 (11)	36		24	
Mucinous			0.02		0.01
Aneuploid	1(1)	7*		3*	
Diploid	13 (12)	70		61	
Endometrioid			0.6		0.3
Aneuploid	5 (4)	75		56	
Diploid	20 (18)	73		68	
Clear cell			0.6		0.4
Aneuploid	2 (2)	22*		11*	
Diploid	5 (4)	58*		57*	
Missing	1 (1)				

Table 26: continued

Tumor factor	Total n=112(%)	5 Year survival rate	P value	5 Year RFS rate	P value
Tumor size					
</= 12.0 cm			0.2		0.2
Aneuploid	20 (18)	31		30	
Diploid	30 (27)	54		40	
> 12.0 cm			0.3		0.2
Aneuploid	12 (11)	40		40	
Diploid	45 (40)	72		68	
Missing	5 (4)				

* = Mean survival, month, RFS: relapse free survival.

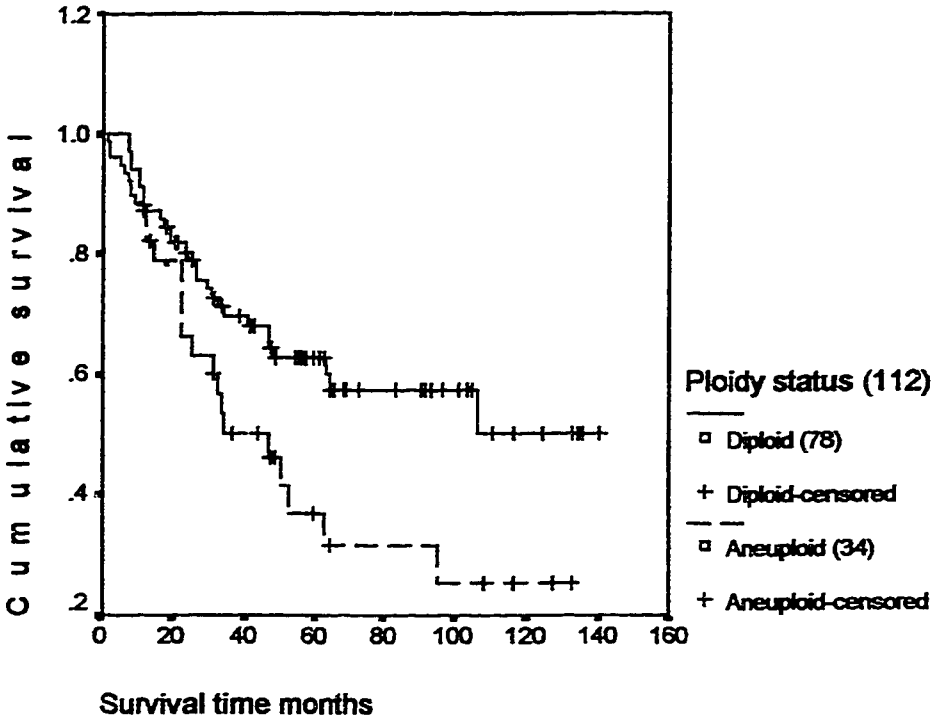

Figure 27: Patients with aneuploid tumor (34 case, 30%) had lower survival rates than patients with DNA diploid content tumors (78 cases, 70%), *p = 0.03.*

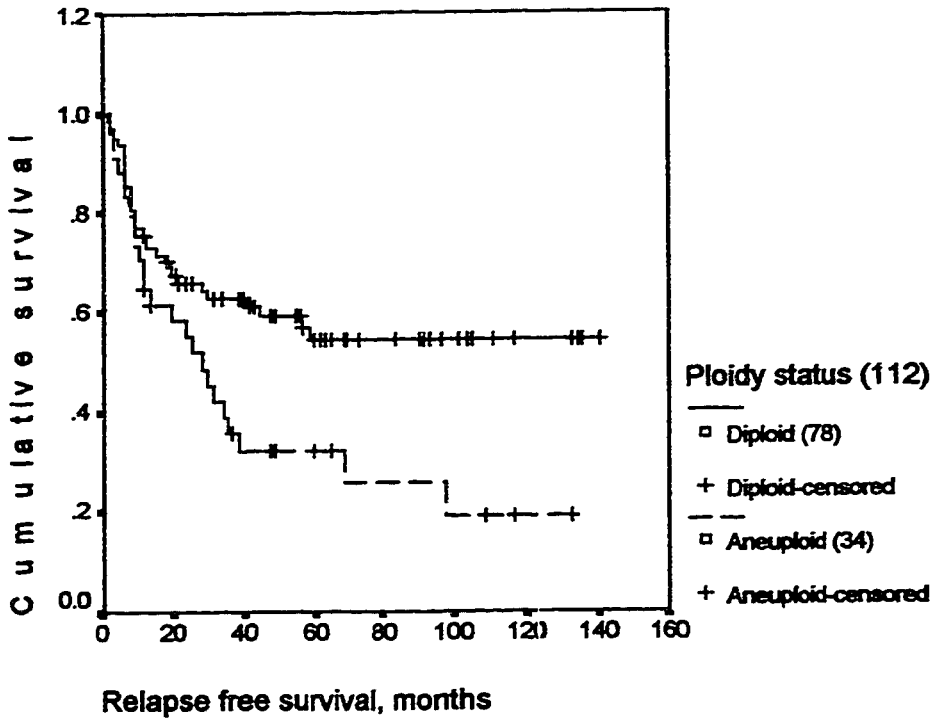

Figure 28: Relapse-free survival rates were significantly different in patients with diploid DNA content compared to patients with aneuploid tumors, $p = 0.01$.

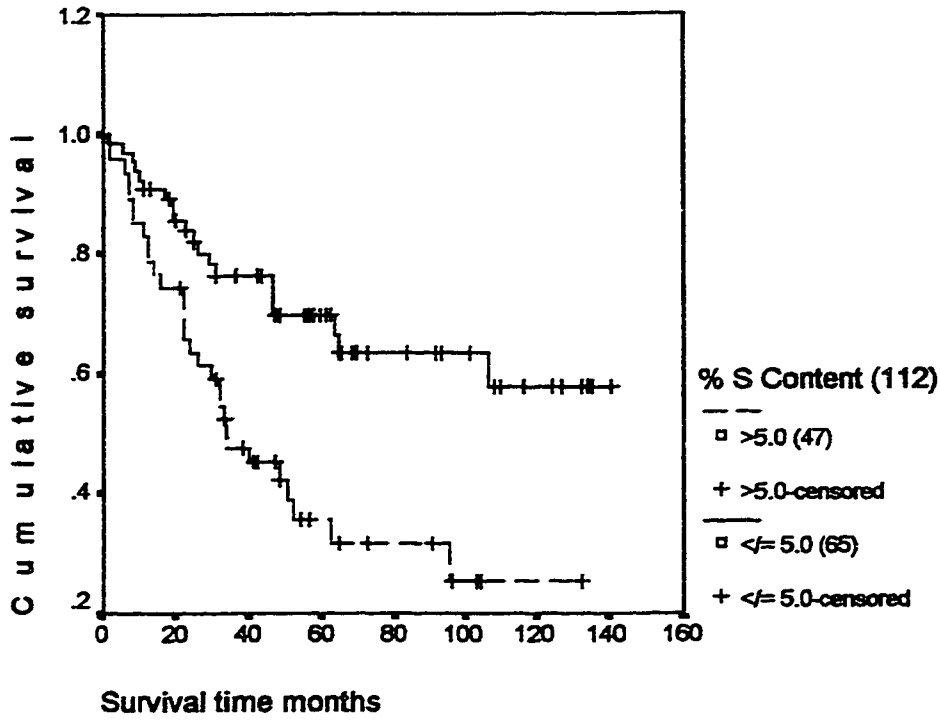

Figure 29: Survival curves for <5.0% S content (65 cases, 58%) was significantly different from >5.0% S content (47 cases, 42%), *p = 0.0008*.

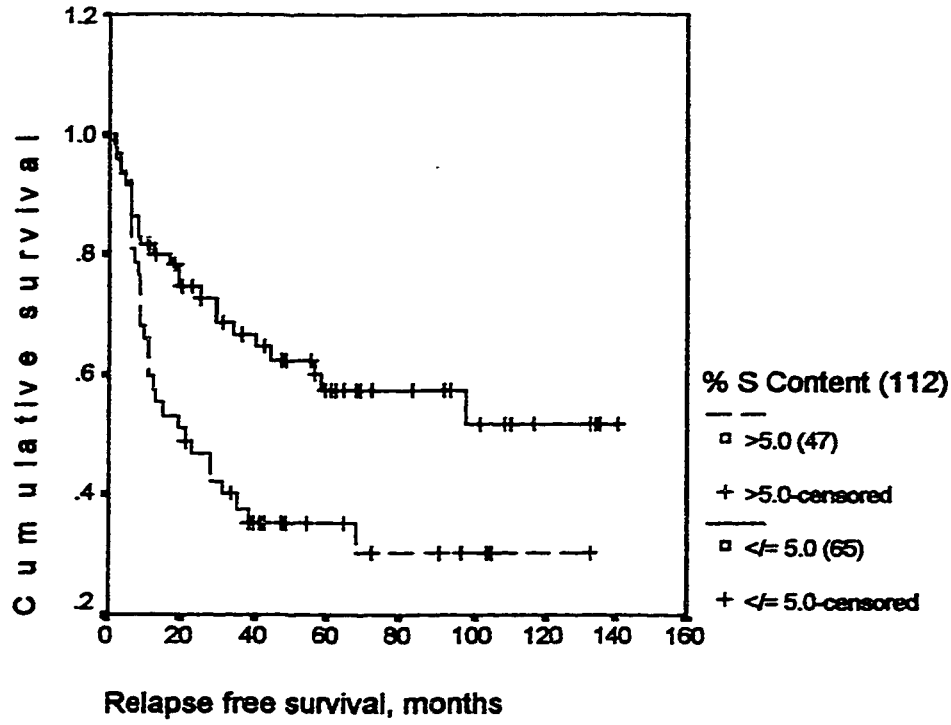

Figure 30: Relapse free survival rates in relation to %S content. The patients in the high % S content group (>5.0) had a shorter relapse free survival than did those with the low % S content group (<5.0), *p = 0.004*.

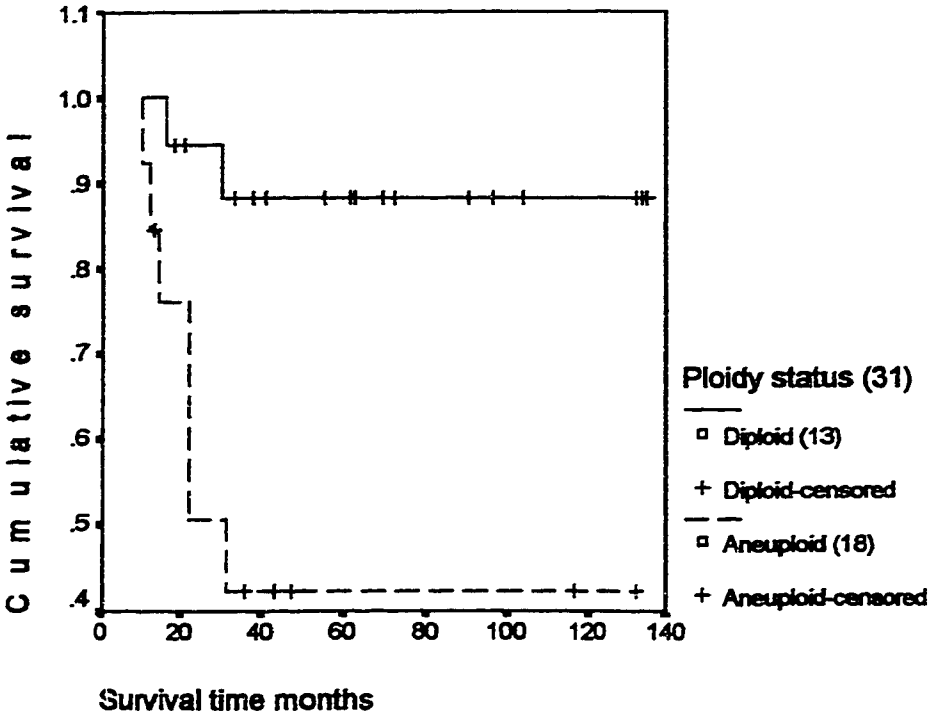

Figure 31: Survival rates within grade 2 cases grouped according to DNA content. The aneuploid tumors had significantly lower rates of survival than diploid tumors, *p = 0.007.*

Since there were at least 70% (78 cases) of the cases with diploid DNA content we subgrouped the cases with <5.0% S content, and >5.0% S content into low risk and high risk categories. Table 27 indicates that the number of patients with diploid DNA content, and low S phase content were twice the number of cases with the high risk S content category. The significance of dividing the diploid patients into subsets based on % S content indicated the influence on survival in ovarian cancer patients, Figure 32, $p = 0.006$.

Table 27: EOC patients, survival rates association of diploid cases with % S phase content, and high S % content with ploidy.

Flow cytometry	Total	5 Year survival rate	P value	5 Year RFS rate	P value
Diploid	n=78(%)		0.006		0.03
</= 5.0% S content	51 (65)	75		62	
> 5.0% S content	27 (35)	40		43	
> 5.0% S Content	n=47(%)		0.4		0.3
Aneuploid	20 (42)	*		23	
Diploid	27 (58)	*		42	

* Censored observations, log rank test.

Cox's regression analysis of clinical (Residual disease, stage, and histologic type) and molecular markers (P53 overexpression, DNA content, and % S phase content) identified residual disease as independent factor related to survival and residual disease as well as stage related to relapse free survival in 100 ovarian cancer cases available for analysis (Table 35).

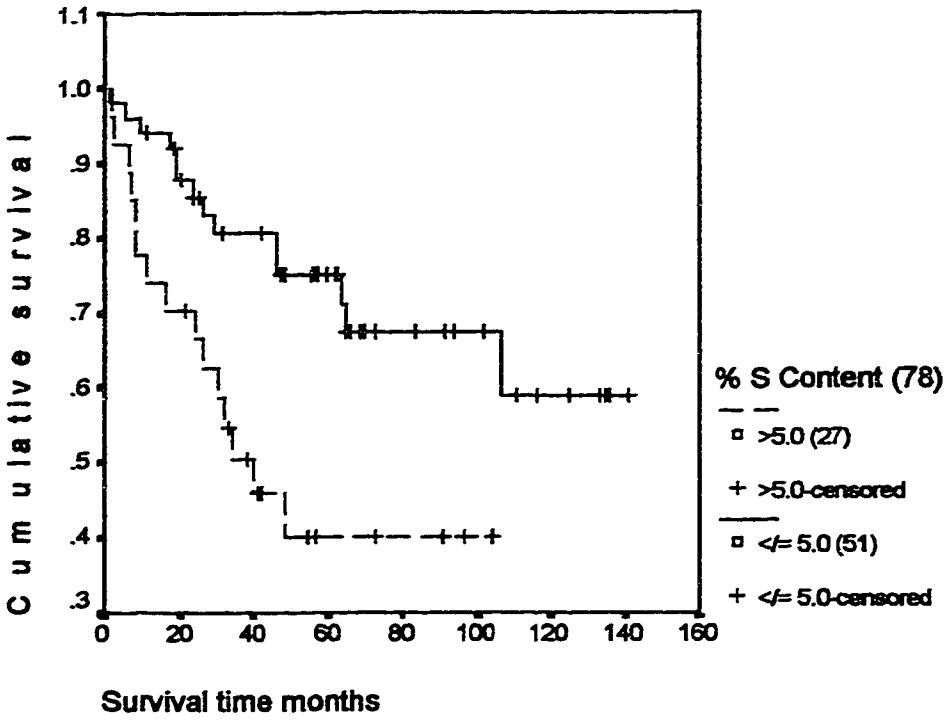

Figure 32: Survival rates within diploid cases that had <5.0% S content (51 cases, 65%) was significantly higher than the cases with >5.0% S content (27 cases, 35%), *p = 0.006.*

4.6 Evaluation of microsatellite instability studies in primary ovarian cancer cases

The results of RER are summarized in Table 28. Figure 33, the upper electropherogram represents amplification of normal tissue and the lower electropherogram represents amplification from tumor tissue at p53 dinucleotide loci. The decision to classify a loci as being affected by RER+, came from the extensive production of extra bands, and extending several repeat units in the tumor as compared from the normal alleles.

Of the eighteen patients analyzed for microsatellite instability at least nine patients showed instability at one or more loci. A case was considered to manifest RER+ if more than one loci was showing microsatellite instability. In this study there were two RER+ tumors. Both the cases with RER+ phenotype were with ES disease. In the RER- phenotype, data on stage was missing in one case, four cases were with AS and two cases with ES.

P53 status was found to be negative in the two (RER+) cases. Among the (RER-) 78% (7/9) cases, and 57% (4/7) cases were p53 negative and the remaining three cases overexpressed p53. Data on DNA content was available on sixteen cases. Aneuploid DNA content was noted in the two RER+ tumors. Of the remaining cases with RER- phenotype, 60% (3/5) were aneuploid and 40% (2/5) were diploid.

The histology of the two RER+ tumor was serous and undifferentiated type. Serous histologic type was the most common, 5/7 among RER- tumors. There was one case each of endometrioid and clear cell histology in the remaining two RER- tumors. Grade of the two RER+ tumors was of moderately and undifferentiated nature. There

were 4/7 undifferentiated, 1/7 moderately differentiated and 2/7 well differentiated tumors in the RER- category.

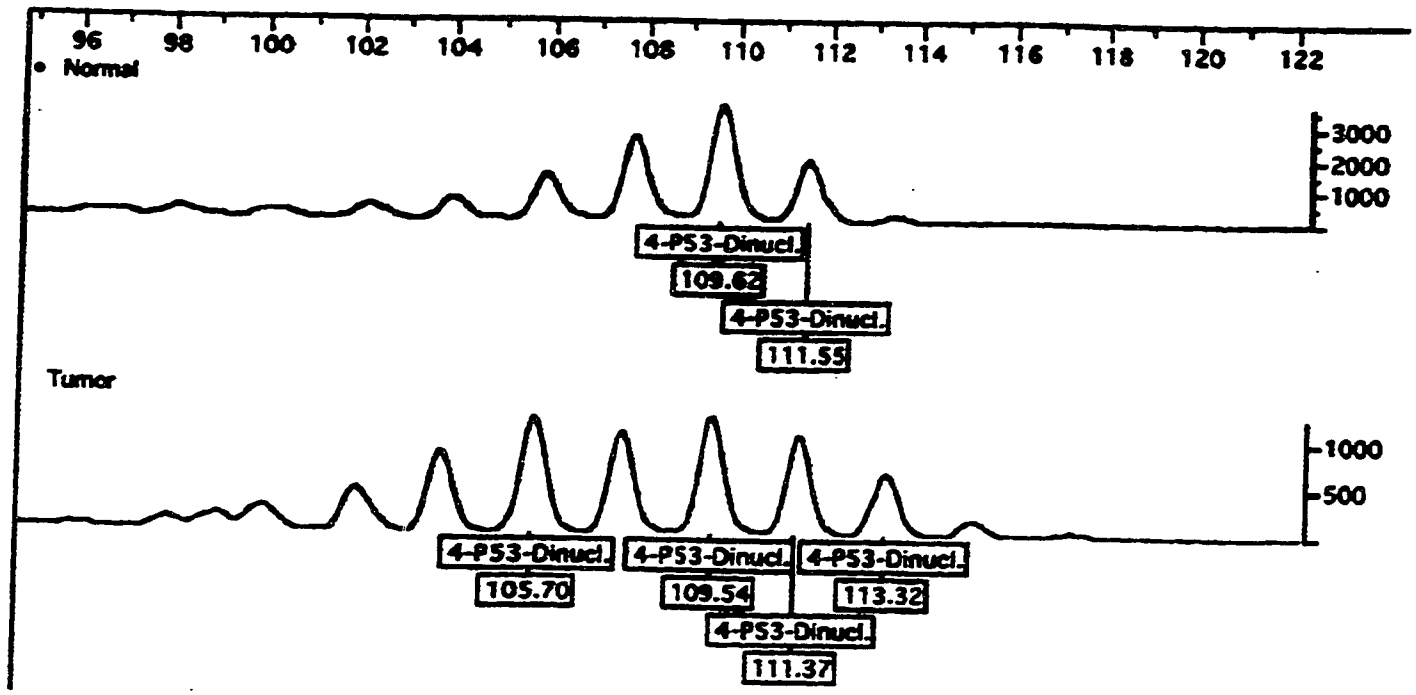

Figure 33: The electropherogram shows the microsatellite instability at the dinucleotide repeat marker P53-dinucleotide from a normal and tumor DNA pair in ovarian cancer patient specimen, a homozygous allele.

Table 28: Results of molecular changes at nine loci in ovarian carcinoma patient cases.

Case #	NM23	D18S35 DCC	TP53-DI	D5S346 APC	TP53-Penta	D2S123 MSH2	D1S2883 HPC1	D3S1611 MLH1	D7S501 MET	RER	DNA status	P53	Hist. Type	Stage	Grade	Adverse outcome
1	4	3	4	1	3	4	1	0	4	P	A	N	S	ES	M	0
2	1	1	2	0	1	1	0	1	1		D	P	E	AS	U	0
3	3	0	0	2	1	0	1	0	0		A	P	S	AS	U	0
4	0	3	4	3	2	0	0	0	1	N		N	E	-	U	0
5	1	0	1	3	1	1	1	3	1		D	P	S	AS	M	1
6	1	3	1	1	1	2	1	1	0		A	N	U	ES	U	0
7	0	1	0	3	3	1	0	0	0		A	N	S	AS	U	0
8	1	3	0	0	3	2	1	0	0			N	E	AS	U	1
9	0	2	4	1	2	0	4	0	4	P	A	N	U	ES	U	0
10	1	0	4	2	2	2	2	1	0	N		P	S	AS	U	0
11	0	1	3	2	2	2	2	1	0			P	S	-	U	1
12	1	4	0	2	1	1	2	1	0	N	A	N	C	ES	M	0
13	1	2	4	2	2	2	1	0	1	N	A	N	S	AS	W	0
14	1	2	2	2	1	2	4	1	1	N	D	P	S	AS	U	1
15	2	1	3	2	2	3	3	1	4	N	A	N	S	AS	W	0
16	0	3	0	2	1	0	0	1	3		A	N	E	ES	U	0
17	1	3	1	2	1	2	0	1	4	N	D	P	S	ES	U	0
18	0	0	0	2	0	3	0	0	0		A	N	S	AS	W	1

0: LOH negative, 1: LOH, 2: failed reaction, 3: non-informative, 4: RER: replication error, P: positive, N: negative, A: aneuploid, D: diploid, Hist. type: histologic type: C: clear cell, E: endometrioid, S: serous, U: undifferentiated, AS: advanced stage, ES: Early stage, W: well differentiated, M: moderately differentiated, U: undifferentiated.

An example of LOH at marker NM23 is shown in figure 34. The peak heights in fluorescence units are shown on the y axis on the right. The upper electropherogram from normal tissue; lower electropherogram from tumor tissue. The label below each peak is size in bp.

Results of LOH for nine markers and outcome events of the eighteen patients with primary ovarian carcinoma are given in Table 29. The frequency of LOH at the nine loci were: NM23-H1: 60% (9/15), D18S35: 50% (4/8), TP53-Di: 33% (3/9), D5S346: 60% (3/5), TP53: 89% (8/9), D2S123: 50% (4/8), D1S2883: 50% (6/12), D3S1611: 53% 9/17), and D7S501: 38% (5/13).

There were some cases in which reactions failed to amplify the DNA. LOH studies using microsatellite markers enable identification of loci with MSI (Aaltonen et al., 1993, and Thibodeau et al., 1993). The cases in which microsatellite instability occurred were not included in determining LOH frequency. In addition cases which were homozygous were denoted as non-informative.

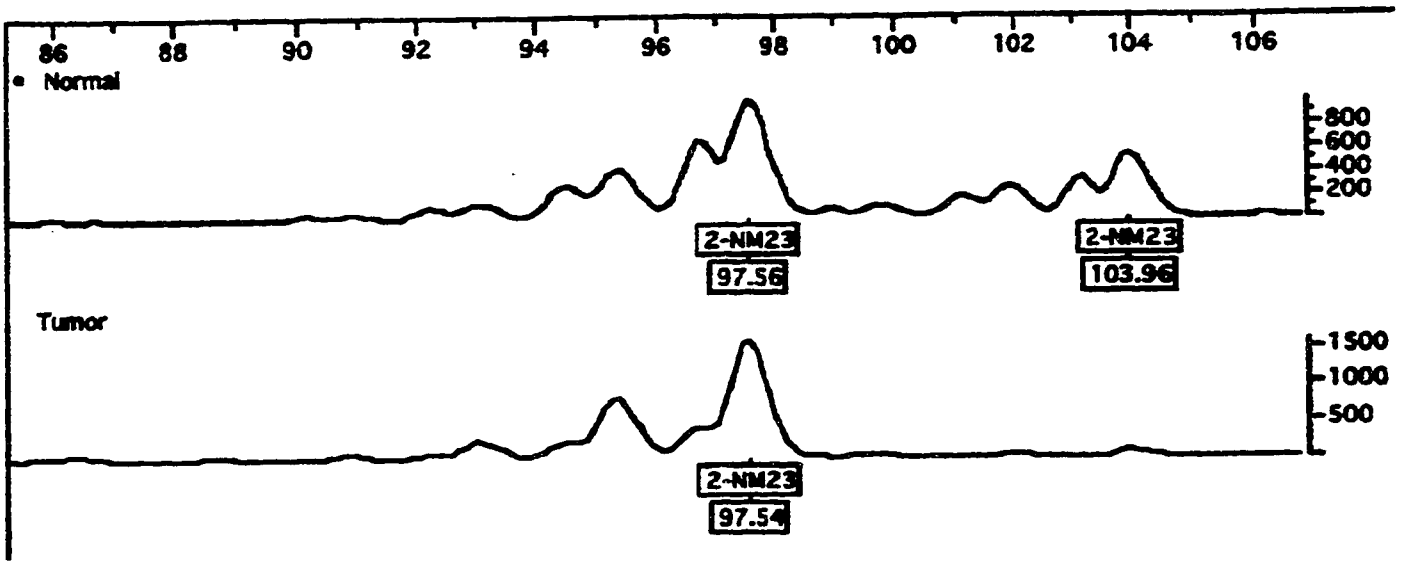

Figure 34: The first electropherogram represents the normal DNA sample and the second electropherogram to the tumor DNA sample. An example of LOH at the *NM23-HI repeat locus in ovarian cancer patient specimen.

Furthermore data on LOH at the nine loci was analyzed to determine an association with P53 overexpression and DNA content as shown in Table 29. P53 immunoreactivity was not found to be associated with LOH at any of the nine loci. Association between DNA content and LOH data at loci D7S501, $p = 0.05$ was the most significant out of all the nine loci analyzed for associations.

Table 29: Summary of the markerwise comparison of results of LOH at each loci with P53 immunoreactivity and DNA content.

Locus name	LOH	P53 # of Cases		P value	DNA content # of Cases		P value
		N	P		Aneuploid	Diploid	
NM23-HI	N	5	1	0.3		4	0.2
	P	4	5		4	3	
D18S35	N	1	3	1.0	1	2	1.0
	P	2	2		1	2	
TP53-Di	N	5	1	0.2		5	0.1
	P	1	2		2	1	
D5S346	N	1	1	0.4	1		0.2
	P	3				3	
TP53-Penta	N	1		0.4		1	1.0
	P	3	5		4	4	
D2S123	N	3	1	1.0		3	0.4
	P	2	2		2	2	
D1S2883	N	4	2	1.0	2	3	1.0
	P	4	2		1	4	
D3S1611	N	7	1	0.4		6	0.2
	P	4	5		3	4	
D7S501	N	5	3	0.6		5	0.05
	P	2	3		3	1	

N: negative, and P: positive.

Chapter 5

5.0 Prognostic value of patient treatment, tumor and tumor specific factors in ovarian cancer

EOC is the most common form of ovarian carcinoma, accounting for approximately 90%, of the malignant tumors arising in ovary. It is thought that EOC arises from malignant transformation of the coelomic epithelium. The incidence of ovarian tumors increases with age, the median age of diagnosis is 63 years and the highest incidence occurs in patients in their eighth decade (Yancik et al., 1993). The median age of the patients was 58 years (range, 18 - 84 years) in our study, were similar to a large series by Dembo et al., 1982. In the current study age was associated with overall length of survival ($p = 0.04$) or but not with shorter RFS ($p = 0.07$). Younger women have been reported to have a lower risk of relapse which was attributed to the disease factors including early stage, well differentiated tumors and minimal residual disease after laparotomy (Dembo et al., 1982a). In our investigation out of the 102 patients under 58 years the most important factor predicting risk of relapse for 69 cases available for analysis, by Cox's proportional hazard model after adjusting for grade and residual disease was AS. In contrast, for women above 57 years, 72 cases available out of 105 cases, we found grade and residual disease to be the most significant predictor of risk of relapse after correcting for grade and stage. Other studies have shown that although age was an important prognostic indicator by univariate analysis, in a study of 560 women, it did not remain an independent prognostic factor associated with survival by multivariate analysis (Swenerton et al., 1985).

Previously, good performance status as determined by ECOG scale is of value in identifying patients who are likely to have a favorable treatment outcome and less likely to develop toxicity effects (Swenerton et al., 1985, and Klein et al., 1985). In a recent study, Ochiai et al., 1994, also indicated that performance status was associated with survival in ovarian cancer patients. Taken together these results of performance status association to outcome further emphasize the significance of noting the ECOG status of the patient at the initiation of treatment. Data regarding performance status was available only for 40% of the patients in this series, 57 cases were classified ECOG score 0 and the others had scores between 1 and 4 (Table 8 and 9). Despite the limited number of cases with data on performance status, this variable was significantly correlated to overall survival and RFS.

Data in the literature indicates the FIGO staging system is useful in predicting 5 year survival (FIGO, 1979). Cumulative series have predicted a 5 year survival of 50-85% for stage I, 37-79% for stage II, 7-16% for stage III and 2-8% for stage IV (Swenerton et al., 1985, Klein et al., 1985, FIGO, 1979, Sevelda et al., 1990, and Sigurdsson, 1983). In the current study, ES (86 cases, 42%) patients had a median length of survival of 46 months, survival rate at 5 years was 82% in comparison to AS (108 cases, 52%) patients with median overall survival of 25 months, survival rate at 5 years being 22% ($p < 0.001$). The five year survival rates for the EOC patients were: 82% stage I, 85% stage II, 27% stage III and <1% stage IV. Five year survival seen in this series is similar to other series. The presence of adhesions at the time of surgery were evaluated, by univariate analysis, and found to be significantly correlated to both reduced survival and RFS. Dense adherence has been previously associated with increased risk of relapse equivalent to stage II (Sevelda, 1990, and Dembo et al., 1990).

Histologic grading is based on either on the degree of differentiation, (Day et al., 1975) or presence of nuclear pleomorphism and the number of undifferentiated cells on tumors (Decker et al., 1975). Grading has prognostic importance in ovarian tumors but is not considered to be an independent prognostic marker (Baak et al., 1987, Cramer et al., 1987, and Stalsberg et al., 1988). Since the reproducibility of grading ovarian cancer is somewhat limited with a high degree of intra- and inter-observer variation, (Hernandez et al., 1984), this perhaps contributes to grade not being established as an independent prognostic factor, overall stage supersedes grade prognostic significance. Patients with well differentiated tumors are reported to have a 70% 10-year survival, moderately differentiated tumors a 30% 10-year survival, and poorly differentiated tumors a 5% 10-year survival (Sorbe et al., 1982). The 10 year survival rates for the three histologic grades from the current series indicated 72% for well differentiated and 42% for moderately differentiated carcinomas. The patients with poorly differentiated cancers had a higher 5 year survival rate of 18% in our population when compared to a previous literature report (Sorbe et al., 1982). In this retrospective series, the 41 cases with well differentiated and 52 cases with moderately differentiated carcinomas had mean survival time of 109 and 85 months respectively whereas 52 months for poorly differentiated cancers. There was a statistically significant difference in overall ($p < 0.001$) as well as pairwise RFS between patients in the three histologic grade categories.

It has been established that the most common histologic type of EOCs are serous carcinomas accounting for nearly 40% to 70% of cases (Russell, 1979a, and Russell, 1979b). Serous ovarian carcinomas are frequently disseminated at diagnosis (Aure et al., 1971). About 50% of the serous carcinomas cases are bilateral (Patricia, 1993). The

overall 5-year survival for serous ovarian carcinoma patients has been reported between 20% to 35% (Sorbe et al., 1982, and Aure et al., 1971) This study concurred with other reports with serous carcinomas accounting for 42% cases. Bilateral ovarian involvement was seen in 67% of these cases with an overall 5 year survival of 41%. Nearly 50% of mucinous carcinomas present at an ES thus the survival of these patients is relatively favorable (Sorbe et al., 1982, Dembo and Bush, 1982a, and Schray et al., 1983). In the current study, 70% of patients (16 cases) with mucinous histology presented at an ES and had the best prognosis of all the histological types with, median survival time of 110 months. The 5 year survival rate for mucinous carcinomas were 70%. This favorable outcome of patients with mucinous carcinomas correlated with ES of disease presentation in this series. The 5 year survival for all stages of mucinous carcinomas has been reported to be approximately 40% to 60%, with those in stage I having a 5-year survival of 70% to 90% (Aure et al., 1971, and Hart and Norris, 1973). It has been reported that the contralateral ovary is involved in 8-10% of mucinous carcinomas at the time of diagnosis (Yao et al., 1994). For 25 cases of mucinous carcinoma in this study, there were only 32% cases with bilateral ovarian involvement. Endometrioid carcinomas are reported to have much better prognosis than the most aggressive histologic type, serous carcinoma, accounting for 10% to 25% of all epithelial tumors with 40% to 60% of patients surviving more than 5 years (Swenerton et al., 1985, Sevelda et al., 1990, and Czernobilsky et al., 1970a). This may be explained by the fact that the endometrioid tumors are confined to the ovaries in about 50% of patients and bilateral involvement occurs in about 30% to 50% of the patients (Aure et al., 1971, and Czernobilsky et al., 1970a). The patients with endometrioid carcinoma in this study were diagnosed at an ES and fifteen of the thirty

seven had bilateral ovarian involvement with 106 months median survival time and a median RFS of about 68 months. These patients with endometrioid histology had five year survival rate of 67%. Clear cell carcinomas had a median survival of 42 months and accounted for 7% of the epithelial ovarian cancers in this series. The clear cell tumors are reported to represent 5% of epithelial malignancies, and tend to present at an ES (50% in stage I) (Aure et al., 1971, and Czernobilsky et al., 1970a). The 5 year survival rates are intermediate between those of endometrioid and mucinous carcinomas 50% to 70% (Swenerton et al., 1985, and Norris and Robinowitz, 1971). The data presented here was not quite in agreement to other studies because the clear cell histologic type had 5 year survival rate of 41%. Bilateral clear cell tumors have been reported to occur in 40% of cases (Czernobilsky et al., 1970b) however, in our study there was only one case of bilaterality out of the fourteen overall cases with the clear cell histology. The RFS of 31 months was intermediate between endometrioid (median survival time, 68 months) and mucinous carcinoma (median survival time, 33 months). The clear cell carcinomas and undifferentiated carcinomas had a worse prognosis in the current study. Thirty five (17%) undifferentiated carcinomas, had the shortest RFS time of 9 months. The occurrence of undifferentiated carcinomas has been reported to vary from 0.9% to 15% of cases (Marsoni et al., 1990, and Hart, 1981). The literature reports a 5 year survival rate of undifferentiated carcinomas to be 11% to 25% with the mean survival time of 27 months (Aure et al., 1971, Swenerton et al., 1985, and Silva et al., 1991). In this study the undifferentiated carcinomas had a 5 year survival rate of 29% and a median survival time of 23 months. Histology of ovarian cancer has an affect on survival, which decreases in the following sequence, mucinous, endometrioid, serous, clear cell and undifferentiated

(Gallager, 1975 and Ochiai et al., 1994). This trend of decreasing survival among the different types of epithelial ovarian cancer was evident in our results.

Ascites was identified as an independent prognostic factor in only one other study of advanced ovarian cancer (Rodenburg et al., 1987). In the current series there was a significant reduction in both overall survival and RFS for ovarian carcinoma patients with the presence of ascites by univariate analysis as indicated in Table 8 and 9. In addition, there was a significant correlation of the presence or absence of ascites to malignant cells in peritoneal cytology of these patients ($p = 0.02$). The combination of malignant cells in peritoneal cytology with ascites did not have a significantly adverse effect on RFS (median months, 11, $p = 0.1$) compared to ascites without malignant cells (median months, 20). There was no adverse effect on overall survival for patients with tumor rupture and positive peritoneal cytology, $p = 0.5$. However, in this series patients who were NED and had ruptured at the time of laparotomy (17 cases), had a shorter survival and RFS in comparison to patients with NED and no rupture, $p = 0.0008$ and $p < 0.001$ respectively (Table 30). Consequently patients with rupture at the time of laparotomy and NED might have a different to response to treatment.

Table 30: Survival and RFS of ovarian carcinoma patients after laparotomy with NED.

Tumor factor	Cases	Survival, median months	P value	RFS, median months	P value
Rupture			0.0008		<0.001
No	50	130*		128*	
Yes	17	63		34	

*Survival: mean survival in months, RFS: relapse free survival.

The removal of large tumors in advanced ovarian cancer patients has shown to correlate with improvement in survival (Partridge et al., 1992). The data on initial tumor volume in this series demonstrated that larger tumors (>12 cm in diameter) had a significantly better overall survival and RFS (Table 8 and 9), *p=0.004 and p=0.0005*, respectively, than smaller tumors. The survival difference in patients is probably due to the physiological benefits to the patient (Reviewed in Heintz, 1991). Furthermore, the removal of large tumors may enhance the response of the remaining tumor to chemotherapy. It will be interesting in the future to do analysis based on staging schema that also subdivides stage III based on volume of initial tumor, - the 1985 FIGO staging system.

Primarily, it is the bulk of the disease that can be removed at the time of surgery which enables the patient to respond to chemotherapy before the residual cancer cells develop drug resistance. Residual disease as a marker of prognosis has been reported by several authors (Ochiai et al., 1994, reviewed in Levin et al., 1993). Griffiths (1975), first described the concept of cytoreductive surgery advantages in improving survival, suggesting that the residual disease should be less than 1.5 cm. The influence of residual disease on survival has been confirmed in more recent studies and shown to correlate with survival, on subdividing residual disease into (i) microscopic disease, (ii) optimal disease (2cm or less in residual diameter), and, (iii) suboptimal disease (greater than 2cm in diameter of residual disease), (Hoskins, 1994a). In 1975, Griffiths reported that survival was inversely related to the diameter of the residual disease. In this study, overall the patients with NED had a mean survival time of 118 months, with minimal residual and gross residual disease 46 and 19 months median survival time respectively. The finding

that there was decreased overall survival and RFS with increasing residual disease also reflects the more aggressive biology of these tumors or, that a greater number of tumor cells are left behind after surgery, despite NED, that is no visible tumor cells.

The impact of the size of residual disease on survival in stage III ovarian cancer further shows the benefits of cytoreductive surgery. The large residual tumor masses have a likelihood of not receiving chemotherapy (Bruchovsky and Goldie, 1982, and Gunduz, 1979). Furthermore, according to Goldie and Coldman's theory, as the size of the tumor increases, so do the chances of chemoresistant clones. Hoskins et al., 1994b according to GOG definition of suboptimal residual disease (> 1cm) found a statistically significant improvement in survival in patients who had 1-2 cm residual disease, as compared to those having >2 cm residual disease, ($p< 0.01$). Therefore, the most striking feature is the effect on survival unless the residual tumor is less than 2 cm in diameter or at the optimal disease.

As a subset analysis, cases of ovarian carcinoma with stage III were analyzed to determine the importance of residual disease in predicting outcomes. The median survival in stage III patients was 26 months and mean RFS time was 11 months. Overall there were seventy five ovarian carcinoma patients with stage III disease (7 cases NED, 23 cases minimal and 42 cases gross residual disease). In this study the overall log rank test for patients with stage III and "NED" had the best prognosis in comparison to those with stage III and minimal or stage III with gross residual disease, for survival and RFS. Further emphasizing the importance of residual disease as an important clinical indicator for survival and RFS (Figure 35 and 36). There was statistically significant pairwise

difference in survival between patients with NED (mean survival, 82 months) and minimal residual disease (median survival 22 months), $p = 0.01$, and NED and gross residual disease (median survival 25 months) $p = 0.002$, as well as for RFS (NED, mean months = 72 and minimal residual disease, median months = 11), $p = 0.007$ and NED and gross residual disease (median survival 19 months), $p = 0.0004$ respectively. However, there was no significant association with survival and RFS for the cases diagnosed with stage III and minimal compared to gross residual disease, $p = 0.36$ and $p = 0.26$ respectively.

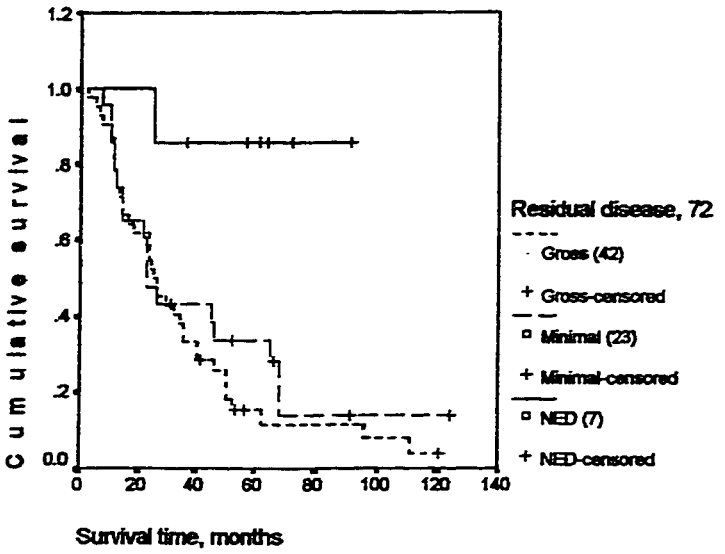

Figure 35: Survival curves of ovarian carcinoma patients in FIGO stage III with residual disease, $p = 0.01$.

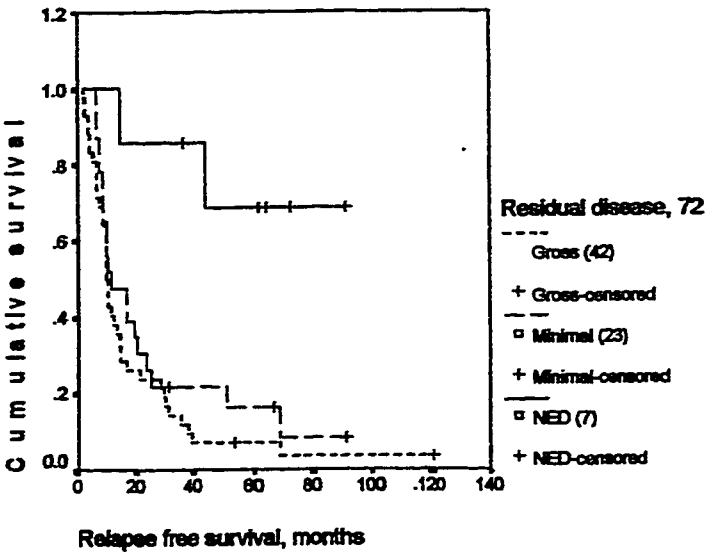

Figure 36: Relapse-free survival estimates of ovarian carcinoma patients in FIGO stage III with residual disease, *p = 0.002.*

A number of variables were identified as the prognostic factors related to survival and RFS by univariate analysis as seen in Table 8 and 9. In the Cox's proportional hazard model, AS, residual disease and histologic type were the prognostic variable that were directly correlated to survival (Table 31). However, prognostic factors related to RFS were residual disease and AS (Table 31). We conclude that any predictive model of ovarian cancer behavior must be built on a foundation that includes the assessment of stage, extent of residual cancer and histologic type following initial surgery.

This study also indicates that tumor rupture in patients that are otherwise free of residual disease is a significantly adverse event with shortened RFS and shortened long term survival justifying its inclusion in staging systems. Positive cytology only appears to be significant in the setting of ascites.

Table 31: Relative risk of survival and relapse in ovarian carcinoma patients.

Tumor factors	N[1]	RR[2] of death	95% CI[3]	P value	N[1]	RR[2] of relapse	95% CI[3]	P value
Stage				0.001				0.0001
ES	77	1	-		77	1	-	
AS	96	4.03	1.75-9.30		101	3.69	1.90-7.16	
Residual disease				0.006				0.0001
NED	67	1	-		67	1	-	
Minimal	42	2.54	1.04-6.20		45	2.61	1.26-5.40	
Gross	64	4.13	1.65-10.32		66	4.67	2.21-9.83	

[1]N: number of cases, [2]Relative risk, [3] Confidence interval.

5.1 CA 125 and CEA prognostic value in patients diagnosed with ovarian carcinoma

CA 125 serum level and FIGO stage have a positive correlation, with an elevated CA 125 level in FIGO stage II - IV patients compared to stage I (Fleuren et al., 1990). The results of our study accordingly indicated a significant association with AS ($p = 0.006$). In addition a positive correlation was found in the immunopositive patients, elevated serum CA 125 level and stage of ovarian cancer ($p = 0.007$), Table 32. However, as such this relationship could not demonstrated in the immunonegative patients ($p = 1.0$), Table 32. A reasonable conclusion of this relationship between stage and serum level is the invasive and/or metastatic attribute of the tumor accompanied with destruction of tissue permeability barriers rather than tumor volume per se (Fleuren et al., 1990).

Table 32: CA 125 antigen immunoreactivity and relationship with CA 125 serum levels with adverse outcome and stage.

Immunohistochemistry	Serum CA125	ES, n=25	AS, n=43	P value
Immunopositive missing(3)				
	0 - 35 u/ml	15	12	0.007
	> 35 u/ml	6	24	
Immunonegative				
	0 - 35 u/ml	3	5	1.0
	> 35 u/ml	1	2	
Immunopositive		Alive, at the end of 5 years	Died within 5 years	
				0.03
	0 - 35 u/ml	10	19	
	> 35 u/ml	3	28	
Immunonegative				
				0.24
	0 - 35 u/ml	4	4	
	> 35 u/ml		3	

ES: early stage, AS: advanced stage.

Table 33: CA 125 serum levels and correlation with clinical outcomes.

Diagnosis	CA 125 serum levels		P value
	0-35 u/ml Cases	>35 u/ml Cases	
Response to therapy			0.01
Yes	39	31	
Progression	4	14	
Missing	1		

Table 34: Trends of CA 125 levels and clinical performance in ovarian carcinoma patients treated with platinum based agents.

Diagnosis	CA 125 Serum levels			
	A Total (44)	B Total (21)	C Total (6)	D Total(18)
Relapse				
No	28	1	1	0
Yes	16	20	5	18
Response to therapy				
Yes	39	18	5	8
Progression	4	3	1	10
Missing	1			
Residual disease				
NED	21	3		2
Minimal	11	9	2	8
Gross	9	8	3	6
Missing	3	1	1	2
Stage				
ES	21	5	1	3
AS	20	15	4	15
Missing	3			

A: 0-35u/ml, B:0-35u/ml and increasing, C: > 35u/ml and D: >35u/ml and increasing, ES: early stage, AS: advanced stage.

Overall, there were 21 out of 25 cases with NED and base line values of CA 125. 20 cases were in ES and the remaining five in AS were with persistent elevated CA 125 marker values (1 case data on stage missing). Response to chemotherapy was obtained in only 20 patients (1 patient no response data missing) and two of the 20 patients have died. In these two patients despite of being NED cases and base line CA 125 values presumably there was microscopic disease remaining at the time of laparotomy. Four of the five the patients with AS combined with NED did not respond to chemotherapy and progressed, had elevated serum levels. NED patients presumably have microscopic disease which are resistant to traditional platinum agents including CA 125 producing tumor cells.

Furthermore, 39 out of 43 patients that responded to chemotherapy had a median time to relapse of 97 months compared to 4 patients who experienced disease progression, Table 33, (median months to relapse = 6, $p < 0.001$). We recommend from our data that those with persistently elevated CA 125 levels be monitored more closely to modify patient management. These observations strongly indicate that CA 125 is a valuable tool in determining patient response to chemotherapy.

The four trends in serum CA 125 levels, Figure 1 and Table 34, and their relevance to clinical performance in ovarian carcinoma can be noted. The > 35 u/ml CA 125 levels and categories, B, C and D demonstrate the usefulness in measuring CA 125 levels. Category B and D with nearly equal percentage of cases (Table 34) indicates a relatively poor response, particularly in category D. The number of patients that relapsed in category B and D also is an indicator of the importance of stratifying patients into the three CA 125 levels when the serum CA 125 level is > 35 u/ml at the beginning of the course of chemotherapy. CA 125 level C had the maximum number of patients with residual disease however 80% (5 out of 6), responded to therapy. Furthermore, from this study the patients in category C would have a favorable outcome in patients treated with platinum based agents.

The prognostic accuracy of the rate of decline of CA 125 level during first line chemotherapy is unclear. In one study, the chance of disease progression was 89% in those patients who had less than seven fold decrease in CA 125 level, in contrast to patients who had 29% probability with a greater than sevenfold decrease after one course of chemotherapy, Fleuren et al., 1990.

In addition, in a study by Mogensen (Mogensen et al., 1992), CA 125 level > 100 u/ml after third cycle of chemotherapy, in ovarian cancer patients, indicated a median survival of 7 months, compared to a 50% 5-year survival for patients with a CA 125 level of 10 units/ml or less. It was also proposed in the same study (Mogensen et al., 1992) that CA 125 measurements may therefore serve to aid in decision making regarding treatment, specifically for cases in which CA 125 level exceeded 100 u/ml one month after the third course of chemotherapy. It has also been suggested that by changing chemotherapy regimens or replacing chemotherapy by palliative therapy on the basis of CA 125 regression, the patient survival and quality of life may be improved as compared to the predetermined and fixed protocols only when effective second line treatments are available (Berkowitz et al., 1993). Monitoring the course of CA 125 post operatively and changing chemotherapy regimens would probably have potential benefits especially in patients with advanced disease who develop resistance to cisplatinum based combination agents but may benefit from receiving agents such as Taxol.

The measurement of CA 125 levels after primary treatment is a non-invasive method for detecting relapse of the disease as indicated by several reports in the literature. Attempts to identify relapse by radiographic and operative procedures often expose an asymptomatic patient to psychological stress and probably jeopardize quality of life.

In the past, a study has shown that patients with normal serum CA 125 levels at the time of relapse had a better prognosis than those with higher values (Makar et al., 1993). Patients in this study, treated with platinum combination regimens, with low serum CA 125 levels had a longer RFS, 5 year survival rate 65%, in comparison to the patients who had elevated CA 125 measurements, 5 year survival rate 8%, $p < 0.001$. The

variables stage, residual disease and histologic type were chosen since these were the only variables significantly associated with survival and RFS in a previous study from this institution (Advikolanu et al., 1996). Consequently multivariate analysis, 78 cases available, indicates CA 125 (RR 7.73, CI: 3.3 - 18.07), and stage, (RR 3.15, CI: 1.31 - 7.59) are the two most independent prognostic variables predicting survival in ovarian carcinoma patients undergoing platinum based regimens.

Overall, normal serum CA 125 and immunoreactivity of the antigen were not correlated in our study ($p = 0.2$). The lack of correlation in our study has been found by several authors (Dietel et al., 1986, Breitenecker et al., 1989, and Motoyama et al., 1990). This observation could explained due to insufficient release of the antigen into circulation (Dietel et al., 1986). In addition, it potentially is due to the relatively low percentage of CA 125 positive cells in some tumors (Kabawat et al., 1983a, Friedlandler et al., 1988, Redman et al., 1988, and Maughan et al., 1988), which would be removed at cytoreductive surgery or eliminated by chemotherapy before the regrowth of the tumor.

Is CA 125 a marker of differentiation? Is it a prognostic marker in this set? Immunohistochemically patients positive and negative tumors CA 125 have a similar disease evolution and survival (Kabawat et al., 1983a, and Friedlander et al., 1988). We have compared the patients with immunopositive staining and serum CA 125 levels. Our results indicate an adverse prognosis, both survival and RFS, in patients with immunopositive and elevated CA 125 levels ($p < 0.001$). In addition, immunonegative cases and serum CA 125 levels were also correlated to prognosis. The fact that there were 3 cases with elevated CA 125 and had negative immunoreactivity could be due to the false negative results with M11 antibody. The negative reactivity in these 3 cases could

also be due to either one of the following factors, poor sampling of tissue for immunoreactivity studies, false positive CA 125 levels or that the residual of tumor cells became CA 125 positive.

There is a lack of knowledge on the function(s) or metabolic regulation of CA 125 is still unknown. The M11 recognizes a high molecular weight subspecies similar to OC 125 and was found to be almost exclusively localized to the extracellular glycocalyx matrix (O'Brien et al., 1991). Furthermore, the development of the M11 antibody recognizing the antigen to the cell surface of tumor cells may provide a basis for more sensitive assay to detect CA 125 in patient sera and improve drug delivery or imaging systems. Although this is a retrospective study and involves seventy one cases to determine the expression of CA 125 on tissue using the M11 antibody, we propose that a concomitant study of tissue expression as well as serum level study would yield a more sensitive test in ovarian carcinoma cases.

In addition CEA overexpression was observed in all mucinous ovarian carcinomas. In the past, similar results of CEA tissue expression in mucinous neoplasms has been reported by several authors (Charpin et al., 1982, and Fenoglio et al., 1981).

We also suggest that the monoclonal antibody, M11, needs to evaluated both as serum marker and tissue expression studies in clinical settings retrospectively and prospectively. Secondly, the M11 antibody needs to be evaluated as tumor marker concurrently with OC 125.

In conclusion therefore, serum CA 125 level measurement is a valuable tool in predicting the RFS and overall survival, in patients with CA 125 producing neoplasms. In our study CA 125 immunopositive tumors and serum CA 125 were related to outcome,

therefore we recommend the use of immunohistochemistry to select the optimal serum markers. Since all the mucinous neoplasms were CEA overexpressors, we suggest the evaluation of CEA expression as a complementary tool in patients with cancers not constitutively expressing CA125.

5.2 General genomic changes

5.2.1 DNA content

The ability of flow cytometry to predict prognosis due to abnormal DNA content and proliferative activity in ovarian carcinomas is so far at an investigative stage.

The studies in the past (Rapi et al., 1996, and Danesi et al., 1997) suggest a requirement for multiple sampling whenever feasible to minimize tumor heterogeneity problems (i.e. ploidy variation such as diploidy and tetraploidy and secondly DNA index). Flow cytometry analysis was used in the current study as a marker of general genomic instability. In this instance 34 (30%) of 112 EOC were found to be aneuploid. The role of abnormal DNA content as an unfavorable prognostic factor has been indicated by several investigators in both prospective (Khoo et al., 1993, Lage et al., 1992, and Iversen et al., 1994) and retrospective studies (Gajewski et al., 1994, Brescia et al., 1990, and Kallioniemi et al., 1988a). The 5 year survival rates of aneuploid DNA content cases was 38%, significantly shorter than for DNA diploid tumors 64% ($p=0.03$). 5 year survival rates have been found to be significantly shorter in DNA aneuploid (22%) tumors than diploid tumors (50%) (Brescia et al., 1990). There are several other investigators that have shown a significantly poor survival in aneuploid tumors correlate increased DNA

content (Gajewski et al., 1994, Blumenfeld et al., 1987, and Friedlander et al., 1984). DNA ploidy status and its association with prognosis in ovarian cancer is elusive at this stage (Resnik et al., 1997, Khoo et al., 1993, Pfisterer 1994, and Schueler et al., 1996). The patient tumor factor such as ES and DNA content showed a significantly poor 5 year RFS rate ($p=0.05$). Although there was a significant association of abnormal DNA content with stage ($p=0.01$), there was no prognostic significance of DNA ploidy in AS patients. This finding may be of significance if found in multivariate model for therapeutic decisions. Albeit, the decision to treat patients with stage Ic and IIc with aggressive therapy is debatable and remains unresolved (Young et al., 1990). With reports in literature indicating up to 20% relapse rate despite treatment (Percolli et al., 1994, Young et al., 1990, and Vergote et al., 1992). In the current study, stage I and II patients were combined due to the limitations of the data available on FIGO substages. Therefore, though DNA content may be used in predicting RFS our data is of limited value without comprehensive data on FIGO substage. DNA content and tumor specific factor such histologic grade 2 was correlated with adverse overall survival ($p=0.007$), and RFS ($p=0.002$) (Table 26).

AS disease and DNA content were not associated with survival and RFS (Table 26). The results of flow cytometry measurement in advanced ovarian cancer patients to predict outcome are conflicting. Blumenfeld et al., 1987 reported ploidy as a significant prognostic factor however, Barnabei et al., 1990 found S-phase fraction to be more significant in predicting survival. In most recent studies, ploidy was not of independent prognostic significance (Gajewski et al., 1994). Furthermore, in predicting overall survival, the measurement of DNA content in advanced ovarian cancer may be of limited

value unless patients are disease-free after chemotherapy. The use of S phase fraction may represent an additional tool in these patients to evaluate response to chemotherapy (Braly and Klevecz, 1993).

The grade 1 and 3 tumors and their association with DNA content was not a predictor of RFS or overall survival. In a retrospective study, Brescia et al., 1990, showed almost equal distribution of ploidy pattern in grade 1, 2 and 3 by degree of differentiation with exception of grade 1 tumors that were 70% diploid. Histologic grade was also determined by nuclear pleomorphism in the study by Brescia et al., 1990, and indicated a similar distribution of grade 2 and 3 tumors among DNA diploid and aneuploid tumors. Grade however, was predictor of shortened 5 year survival rate in Brescia et al., 1990 study (grade 1 = 65%, grade 2, and 3 = 26%). The results of the current study in grade 2 ovarian cancer patients also was indicative of shortened RFS and overall survival, Table 26. In Grade 2 tumors there were 42% aneuploid and 58% diploid cases with significant survival (Figure 31), and RFS differences in 5 survival rate, $p=0.007$, and $p=0.002$ respectively. The significance of this finding is the importance of keeping grade 2 tumors as a distinct category while analyzing data to predict outcome in ovarian carcinoma patients, though some studies in the past on ploidy status have combined grade 2 and 3 tumors (Gajewski et al., 1994, Blumenfeld et al., 1987, Kallioniemi et al., 1988a, and Rodenberg et al., 1988).

Aneuploid content and serous histologic type showed a trend towards poor overall survival and RFS (Table 26) $p=0.06$, and $p=0.07$. However, DNA content and histological type are not correlated (Brescia et al., 1990). In this study, the patients with residual disease there was no association of abnormal DNA content to survival or RFS.

The majority of data on DNA content in the literature indicate a statistically significant association with bulky residual disease (Pfisterer et al., 1994, Khoo et al., 1993, Rodenburg et al., 1987).

Survival and % S phase content that have been examined in several studies found a shorter survival with a high rather than a low % S phase content (Barnabei et al., 1990, and Kallioneimi et al., 1988b). The % S phase content that was used to categorized between prognostic groups varied from one study to the other. The data on the proliferative capacity (% S phase content) also demonstrated the significantly shortened survival among the high % S content cases ($p=0.0008$).

5.2.2 Microsatellite instability

Table 28 shows the eighteen cases investigated to determine the presence of RER and LOH phenotype in ovarian tumors. In addition, we also determined the ploidy status and P53 expression of these eighteen cases (Table 28).

The two RER+ tumors had aneuploid DNA content and in addition also were p53 negative and in ES (Table 28). In one case the RER+ tumor had MSI at nm23-H1, P53-di, D2S123, and D7501, the second RER+ tumor had MSI at P53-di, D1S2883, and D7501. MSI at p53-dinucleotide, and D7S501 were common to the two tumors. 29%(2/9) tumors were RER+ indicating in ovarian carcinogenesis RER+ tumors occur, though the significance of this finding may be better determined in a larger study inclusive of family history.

King et al., 1995 observed MSI in 17% (7/41), both familial ovarian cancers as well as in patients without familial disease. Studies in the past, Osborne and Leech, 1994 examined 25 sporadic epithelial ovarian tumors and detected only 2 alterations among the 1700 repetitive sequences. In one study, it was shown a lack of any MSI in any of the 60 sporadic tumors (Dodson et al., 1993). However, Han et al., 1993, and Wooster et al., 1994 observed 16% (3/19) in dinucleotide repeat and 2/20 in tri and tetranucleotide repeat sequences, though data on histologic classification was not available in these two studies. In contrast, King et al., 1995 study however, included data on histological type, FIGO stage, and MSI was performed on PET using radiolabelled PCR. In the same study, stage I tumors had 75% (3/4), MSI was significantly higher than the other stages 11% (4/37). These studies suggest MSI may be occurring in ovarian cancer with diverse genetic origin, although these inferences of alterations were based on di and tetranucleotide repeats. These findings of positive association between MSI alterations to ES patients (King et al., 1995) are similar to reports on colorectal cancer and favorable prognosis (Lothe et al., 1993, and Thibodeau et al., 1993).

In one study, ovarian cancer, the MSH2 gene was found to be altered in 3/8 cases (Fujita et al., 1995). In RER+ tumors and one of these patient with MSH2 alteration also had LOH at the MSH2 locus (Fujita et al., 1995). Furthermore in the same study, non-germ line mutation in the coding region of MSH2 gene was considered to play an important role in the etiologies of endometrioid carcinoma. In this line, our data on patient survival and RFS might be useful if in the future studies involving determination of mutations are carried out (Single stranded polymorphism and sequencing) in the MSH2 gene. In the present investigation on ovarian carcinomas, RER+ phenotypes had

aneuploid DNA content and were p53 immunonegative. This finding is significantly different than the cases with HNPCC syndrome and sporadic colon carcinoma (Forster et al., 1998). Colon tumors of the right side are characterized by the pattern of diploid DNA content, p53 negative, and RER positivity (Forster et al., 1998). The concept of the presence of wild type p53 and cell cycle arrest in the G1-S phase for activation of DNA repair mechanisms is well recognized.

In this study, among the MSI negative tumors 78% (7/9), p53 negativity and aneuploidy occurred simultaneously in three cases, p53 overexpression and diploid DNA content occurred in two cases, and data on ploidy was not available in two cases. These results are quite different from the colon cancer multistep model where p53 mutations occur as late events. Based on these results it may be speculated that MSI, lack of p53 overexpression and aneuploidy may be characteristic of a subset of ovarian carcinoma cases. Secondly, it may suggest that RER positivity develops as an incidental finding in the pathway of ovarian cancer development as an "epiphenomenon". Moreover, these observations from nine ovarian cancer cases examined with MSI at one of the loci may be different from colon cancer due to tissue type differences for specific gene inactivation and general genomic alterations in human cancer. These include the high expression of nm23 which correlates with carcinogenesis or progression in colon carcinomas (Haut et al., 1991) and neuroblastomas (Hailat et al., 1991). Furthermore, breast carcinoma, (Hennessy et al., 1991) and gastric carcinoma (Nakayama et al., 1993) the reduced expression of nm23 has shown to be associated with the presence of metastasis and/or poor prognosis. Reduction of nm23-H1 mRNA expression levels in one study were associated with lymph node and/or distant metastasis in ovarian carcinomas (Mandi et al.,

1994). In a recent study, Scambia et al., 1996 assessed the nm23-H1 prognostic role in ovarian carcinoma, and nm23-H1 positive cases had significantly higher progression-free survival than nm23-H1 negative cases ($p=0.005$).

5.3 G1-S control genes in ovarian cancer

Cyclin D1-CDK4-p16^{INK4A}-RB-1 genes are functionally interconnected and are components that are regulated transition of cells from mid to late G1 phase into the S-phase (reviewed in section 1.4.3.4) (Figure 1). Wild type p53 controls G1-S phase of the cell cycle by inducing p21$^{WAF1/CIP1}$ protein overexpression upon DNA damage (El-Diery, 1993). These genes that are functionally interconnected in transition of the cells through the G1-S phase of the cell cycle have been investigated in the current study by immunoreactivity studies (p16^{INK4A}, p21$^{WAF1/CIP1}$, and p53) and dot blot hybridization (CyclinD1-CDK4-p16^{INK4A}-RB-1). We were interested in determining the significance of these genes in predicting outcome in primary ovarian carcinoma patients.

Because dysregulation of proliferation is thought to be fundamental to the genesis of carcinoma we expect that the regulations of cell cycle division may be important targets of mutations in a wide array of neoplasms including ovarian carcinomas.

Cyclin D1 is encoded by the CCND1 gene on chromosome 11q13 and acts as growth factor sensor (Sherr, 1993). Thus, deregulation of Cyclin D1 synthesis leads to the cell being less dependent on growth factors and that contribute to oncogenesis. Cyclin D1 is overexpressed in parathyroid adenomas, centrocytic B cell lymphomas, in breast, gastric and esophageal carcinoma.

In the G1-S phase of the cell cycle, Cyclin D1 regulates the tumor suppressor protein, RB1, thereby playing a key role in tumorigenesis. RB1 remains hypophosphorylated throughout G1 phase, is phosphorylated just before S phase, and remains phosphorylated until late mitosis. CyclinD1-CDK4 complex phosphorylates RB1 in late G1. The exclusive role of Cyclin D1 is to phosphorylate RB1 so that the cells enter S phase to replicate their DNA. Cyclin D1 transcriptional activation and RB1 phosphorylation form a negative feed back loop to terminate Cyclin D1 expression.

Presumably the deregulation of D-type Cyclin synthesis would cause continuous activation of the cell cycle and lack of differentiation. The fact that we found only ten cases of Cyclin D1 amplification suggests that this occurs in some but not many ovarian cancers. Courjal et al., 1997 showed Cyclin D1 was rarely amplified 3.3%. However, the lack of Cyclin D1 amplification does not preclude its involvement in ovarian tumorigenesis as alternative mechanisms (overexpression) may cause activation, (Worsley et al., 1997). There were Cyclin D1 deletions in 23 cases and RB1 gene amplification in 19 cases, and deletion in 19 cases by dot-blot hybridization.

CDK4, the partner of Cyclin D1 might also be expected to play a role in G1-S progression and oncogenesis of the ovaries. CDK4 is located on human chromosome 12q13 which also contains the GL1 and MDM2 proto-oncogenes. Amplifications have been reported in the CDK4 gene in human tumors includes gliomas and sarcomas (Khatib et al., 1993). CDK4 overexpression possibly renders some cells insensitive to arrest by TGFβ. In our study, we found that there were 7 cases of amplification, 28 cases of deletions of CDK4 gene. CDK4 may be involved in a subset of human cancer.

Inhibitors of CDK's such as $p16^{INK4A}$ are a novel category of proteins with direct effects on cell cycle regulation and potential involvement in human cancers. The chromosomal position of $p16^{INK4A}$ (9p21) is frequently deleted in a variety of human malignancies. In the current investigation there were 22 cases of $p16^{INK4A}$ deletion for the primary ovarian cancer cases. $P16^{INK4A}$ function may be silenced by alternative mechanisms to homozygous deletion including promoter methylation, mutation or transcriptional repression (reviewed in Sherr and Roberts, 1995, Merlo et al., 1995, Hussussian et al., 19944, Kamb et al., 1994, Mori et al., 1994, and Liu et al., 1995.

The finding of $p16^{INK4A}$ amplification in 7 cases may be of uncertain significance. It may indicate that the 9p21 region may be prone to somatic mutational events, predominantly deletion, but also including amplification.

$P16^{INK4A}$, RB-1, CDK4, or Cyclin D1 gene mutation events were not prognostic factors related to outcome events as shown in Table 16 and 17. In this study, G1-S control genes were evaluated for their prognostic significance in ovarian carcinoma for the first time by dot blot hybridization. The lack of association to outcome events presumably suggests that there may be some other gene (s) that are molecular markers of prognosis in ovarian cancer. Since 94% (59 cases) showed some mutation in $P16^{INK4A}$ - RB1 - CDK4- or RB1. We conclude that G1-S growth control alterations play an important role in ovarian cancer development.

Even though this preliminary study using dot blot hybridization did not show prognostic importance of $P16^{INK4A}$ - RB1 - CDK4- Cyclin D1 genes, it was interesting to assess various markers as prognostic indicators alone or in combination. In ovarian cancer abnormal DNA content has been related to adverse outcome by several investigators (

reviewed in Braly 1992). The current study showed that there was a lack of correlation between $P16^{INK4A}$ - RB1 - CDK4- Cyclin D1 and ploidy status, except with Cyclin D1 amplification and RB-1 deletion (Table 18).

The p53 tumor suppressor gene product was found to be overexpressed in a significant portion of ovarian carcinomas. 18 cases (33%), but we could not find any correlation with mutation in G1-S control genes in the current investigation.

5.4 Specific gene and gene product alterations

5.4.1 Oncogenes in carcinogenesis

5.4.1.1 Erb-B2

The development of malignant cells is due to the uncontrolled cell proliferation involving a number of growth factors and growth factor receptors, signal transduction molecules and oncogenes and tumor suppressor genes (Hollywood and Lemoine, 1992). Previous studies of Erb-B2 amplification suggest upto 32% of cases show amplification of this oncogene and nearly 72% overexpress it in ovarian carcinomas. However, its relationship to outcome in ovarian carcinomas remains inconsistent. In this study, we detected there was no significance of Erb-B2 overexpression in predicting outcome measurements (Table 13). Slamon et al., 1989, studies were one the first in a series of studies that followed in associating abnormalities of Erb-B2 gene, that is amplification and/or overexpression in ovarian carcinoma. In a recent study by Felip et al., 1995, 22% of 106 cases overexpressed Erb-B2 and were correlated with poor outcome, stage, residual tumor as well as response to therapy. The results obtained in the current investigation are different

from the investigators that correlate Erb-B2 overexpression (19%) from PET with significantly worse prognosis, median survival = 20 months, than the Erb-B2 negative group, median survival = 33 months, $p = 0.001$ (Meden et al., 1994). The study by Berchuk et al. 1990, described tumors overexpressing (32%) Erb-B2 have a shorter median survival in patients with advanced ovarian cancer. However, other investigators argue the prognostic significance of Erb-B2 in archival material may be different due to antigen alterations as a result of differences in tissue processing time and fixation procedures.

Erb-B2 overexpression in ovarian carcinomas has been associated with overall decreased survival in some studies in the past, yet there are other investigators showing no relationship to outcome. Haldane et al., 1990, studied 104 archival specimens and only 9% were Erb-B2 overexpressors with no prognostic role. Investigations using fresh tissue report no association of Erb-B2 expression and prognostic significance in ovarian cancer (Huettner et al., 1992, Singleton et al., 1994, and Rubin et al., 1993). The data from this retrospective analysis confirm these above results from various studies and perhaps demonstrate a limited role of Erb-B2 in predicting outcome. However, the conventional tumor factors such as stage and histologic grade were proposed to be better markers of survival and RFS in ovarian cancer patients (Singleton et al., 1994).

5.4.1.2 C-Met

LOH at (D7501) MET proto-oncogene on chromosome 7q31 was detected to be 38% (5/13). Di Renzo et al., 1994 examined the overexpression of C-Met/HGF in ovarian carcinomas by western blot analysis and showed an association with well-differentiated

stage I tumors. The over expression of C-Met/HGF in the study suggest its selection for the ovarian epithelial cells at an early stage in the progression of this cancer. However, it was also noted in the same study, the highest levels of the C-Met/HGF receptor was in pre-menopausal ovarian cancer patients. Although, purely speculative, the Di Renzo et al., 1994 study suggests a different etiologic pathway for ES and AS disease. The fact that there was 38% LOH at this locus, greater than past allelotyping studies probably suggests the involvement of a putative tumor suppressor gene at this locus. Furthermore, a significant association with abnormal DNA content ($p=0.05$, Table 29) suggests general genomic instability in the subset of ovarian cancer cases that were investigated.

5.4.2 Tumor suppressor genes in carcinogenesis

5.4.2.1 $P16^{INK4A}$

The tumor suppressor gene $p16^{INK4A}$ overexpression in 33% (46) of cases did not contribute to adverse survival and RFS in PET from primary ovarian cases, as shown in Table 13. The elucidation of the role in ovarian carcinogenesis has been an elusive goal. Previously, the high frequency (54%) of LOH on chromosome 9p in ovarian carcinomas was first observed by Cliby et al., 1993. In the extensive studies conducted by others (Chenevix-Trench et al., 1993, Osborne and Leach 1994, Rodabaugh et al., 1995, Devlin et al., 1996, and Chenevix-Trench et al., 1997) the high levels of LOH on chromosome 9p were confirmed. The mechanism of $p16^{INK4A}$ inactivation by homozygous deletion and mutation was shown in ovarian cancer lines and primary tumors (Chenevix-Trench et al., 1994, Kamb et al., 1994, and Shih et al., 1997) 14% up to 50% respectively. In studies

analyzing mutations in $p16^{INK4A}$, Marchini et al., 1997, detected no mutations at exons 1 and 2 in 21 primary ovarian tumor DNA samples. Marchini et al., 1997, study detected a lack of $p16^{INK4A}$ expression in 26% (11 out of 42) primary tumors and in three cell lines (OVCAR-5, SKOV-3, and SW626). Furthermore, in the same investigation there was no hypermethylation of $p16^{INK4A}$ promoter region and alternative mechanisms to explain the silencing of $p16^{INK4A}$ expression was suggested. These suggestions are in contradiction to Merlo et al., 1995, and Nishikawa et al., 1995 studies, that have indicated the inactivation of $p16^{INK4A}$ could be due to the methylation of C_pG islands spanning exon 1 and part of exon 2.

In a recent study on PET from epithelial ovarian tumors only 11% were $p16^{INK4A}$ negative, 89% (126/142) showed both nuclear and cytoplasmic staining (Dong et al., 1997). The 5 year survival rate of $p16^{INK4A}$ high and low expression group was 20% and 65% respectively ($p = 0.0004$) in their study, in multivariate analysis $p16^{INK4A}$ high expression was not an independent marker of poor outcome probably due to stronger association of outcome with FIGO stage and grade. High $p16^{INK4A}$ expression in stromal cells in all stages, tumor types and grade was associated with longer survival in the same study. In this context we did not observe $p16^{INK4A}$ stromal reactivity, however the results of $p16^{INK4A}$ immunoreactivity were consistent in nuclear and cytoplasmic expression in some tumors. $P16^{INK4A}$ protein expression in nucleus and cytoplasm in primary human fibroblasts, human cell lines, and paraffin sections has been noted by several investigators (Okamoto et al., 1994, Lukas et al., 1995, and Geradts et al., 1996). The reason for this abnormal localization is not defined, though speculation is that it may be due to its binding

to other unknown oncoprotein in the cytoplasm (Po et al., 1995) and/or mutation at the amino acid region with loss of nuclear localization signal.

5.4.2.2 $P21^{WAF1/CIP1}$

This study shows that in EOC patients $P21^{WAF1/CIP1}$ expression is inversely associated to the P53 protein expression (Table 14). The heterogenous expression of $P21^{WAF1/CIP1}$ in the current study is certainly not an indication of mutation in $P21^{WAF1/CIP1}$ gene, since there are no reports in epithelial tumors (Marchetti, 1995).

Studies on somatic mutations in the gene encoding $P21^{WAF1/CIP1}$ protein show no evidence of mutation in human breast, lung, and ovarian carcinomas (Marchetti, 1995). Also reports on colorectal cancer have shown the absence of mutations in the $P21^{WAF1/CIP1}$ gene (Li, 1995).

$P21^{WAF1/CIP1}$ expression is induced by wild type P53 (El-Diery et al., 1994, and Xiong et al., 1993). In the presence of normal P53, $P21^{WAF1/CIP1}$ expression is induced following radiation or chemically induced DNA damage. The induction of $P21^{WAF1/CIP1}$ causes growth through the inhibition of CDKs, which are necessary for a cell to transit to S phase (El-Diery et al., 1994, and Xiong et al., 1993). The functionally inactive P53 is unable to induce $P21^{WAF1/CIP1}$ protein required for growth arrest and repair of the cell prior to G1-S transition. As indicated by our results, the induction of $P21^{WAF1/CIP1}$ by P53 was not related to these known mechanisms.

The overexpression of P53 and expression of $P21^{WAF1/CIP1}$ in 11 cases suggests the onset of $P21^{WAF1/CIP1}$ induction in these cases through alternative mechanism unrelated to

P53 dependent pathway. Two possible pathways may explain this observation of $P21^{WAF1/CIP1}$ expression:

a) P53 protein which accumulates in the nucleus is mutated, however still preserves some transcriptional activity (Fuchs, 1995).

b) P53 independent pathway of $P21^{WAF1/CIP1}$ induction which has been implicated in ovarian cancer cells as well in other tumor cells (Elbendary et al., 1994, and Chen 1995). Recent evidence of TGF-β mediated induction of $P21^{WAF1/CIP1}$ independent of P53 status probably does not require functional P53 for cell cycle arrest and inhibition of DNA replication (Datto, 1995). The P53 independent induction of $P21^{WAF1/CIP1}$ pathway is retained in neoplastic cells, regardless of the status of P53 (that is, wild type, wild-type inactivated by SV40 T, or mutant) or the state of tumor cells (that is, immortal, tumorigenic, or metastatic). Since the striking feature of P53 is its ubiquitous inactivation in nearly 50% of human cancers (Vogelstein and Kinzler 1992, and Greenblatt et al., 1994), therefore the P53-dependent induction of $P21^{WAF1/CIP1}$ pathway is disrupted in these tumor cells.

5.4.2.3 P53

P53 status was measured in a subset of patients by LOH analysis. The p53 allele loss at the pentanucleotide was 89% (8/9) and significantly higher than at the dinucleotide repeat 33% (3/9). The LOH at dinucleotide repeat locus D17S153 in nearly 65% (20/35) has been described previously (McManus et al., 1996) in AS ovarian carcinoma. Based on our results a lack of association between p53 overexpression and LOH analysis at both loci investigated suggests the presence of a putative tumor suppressor gene (s). This

discordance between p53 expression and LOH suggests that many cases may not be overexpressing p53 but may have allelic deletions on chromosome 17p and q. Allelic deletion on chromosome 17 are the most common in ovarian cancers and both 17p and 17q show losses. It is suspected in addition to p53 at 17p13.1 and BRCA1 at 17q21 there may be more genes at the two loci that act as tumor suppressors or regulators in ovarian cancer (Phillips et al., 1996, Godwin et al., 1994, and Yang-Feng et al., 1993).

The p53 tumor suppressor gene is found to be mutated resulting in increased immunoreactivity in a significant portion of ovarian carcinomas. However, the clinical significance of p53 immunoreactivity is currently controversial. The normal allele of the p53 gene encodes a 53-kD nuclear phosphoprotein involved in the control of cell proliferation. Loss of one of the alleles with loss or mutation of the second allele can lead to deregulated cell proliferation and transformation.

P53 somatic mutation is a common genetic event in a wide variety of tumor types including those of the colon, liver, breast, ovary, and haemotopoietic tissues. In cells with significant DNA damage, normal p53 arrests the cell in the G1 phase and either allows repair before replication or triggers apoptosis. In tumor cells however, p53 is inactivated by mutation or by binding to host or viral proteins so that cells are not arrested in the G1 phase of the cell cycle. It appears that tumor cells containing mutant p53 may be resistant to the cytotoxic effects of chemotherapy (Hickman et al., 1994). Because p53 function is vital to maintenance of genomic integrity, growth and therapy, it is logical that it may play a key role in oncogenesis in tissues of diverse types. Consequently, it may serve as a possible prognostic marker for assessment of disease aggressiveness.

P53 immunofalse positive results may also exist in instances where highly sensitive techniques are used. Or a second possibility is in the event of mechanisms other than point mutation. Similarly, there are several antibodies available from commercial sources and the results from the use of these antibodies varies. Many investigators have observed P53 overexpression in a subset of ovarian carcinomas. However, the inference from these studies are often contraindicatory in defining prognostic significance (Klemi et al., 1995, Hartmann et al., 1994, Kohler et al., 1993, and Sheridan et al., 1994). For example Hartmann et al., 1994, found p53 overexpression in 177 of 284 (62%) patients to be associated with poor survival in ovarian cancer by univariate analysis but not in a multivariate analysis. Interestingly in a recent series of 221 primary EOC P53 aberrant expression was not an independent factor related to survival (Eltabbakh et al., 1996). Taken together, these immunoreactivity studies on p53 expression in ovarian cancer do not implicate its role as an independent prognostic marker.

Understandably, the use of different antibodies, antigen-retrieval techniques, fixation procedures, as well as selection and processing of the specimen most likely are also determinants of the differences in immunoreactivity. In this study, P53 protein overexpression was found in 39% (44 cases) of 112 cases evaluated and a prognostic marker of survival and RFS ($p = 0.04$, and $p = 0.008$ respectively, Table 13).

On the otherhand, P53 overexpression and stage as well as patients with NED/ any residual disease were not found to be associated with survival or RFS. Interestingly, although histopathologic grading is considered as a marker of prognostic significance in ovarian cancer and is a subjective method. In our study, P53 overexpression in grade 2 tumors was a marker of adverse overall prognosis in univariate analysis (survival, p =

0.05, and RFS, $p = 0.01$, Table 21 and figure 21). Klemi et al., 1995 studies p53 overexpression in 44% (60 of 136) cases with ovarian cancer. In addition p53 overexpression was significantly associated with poor histologic grade. However, p53 overexpression and the data on histologic grade was evaluated by combining grade 1 and 2.

In addition, in this study we have complemented the P53 immunoexpression studies with ploidy status and proliferative capacity (S phase content). As shown in Table 20, the p53 immunonegative cases with diploid status and low % S content (</=5.0) were significant prognostic markers of survival and RFS (Figure 19 and 20). Furthermore, the % S content was the most significant factor correlated to adverse outcome (Table 22), $p = 0.001$, followed by abnormal DNA content, $p = 0.008$, and p53 overexpression, $p = 0.01$.

Unfortunately it is not a simple matter to adequately assess p53 function in a malignancy. Simple measurements of nuclear immunoreactivity may produce significant false positive and negative results confounding the analysis.

In the past accumulation of p53 protein in nuclei, mutation in the gene and aneuploidy were significantly correlated (Kihana et al., 1992). Since aneuploidy of tumor DNA is due to chromosomal instability, the p53 protein overexpression and mutational event in the gene could be directly linked with genomic instability. Therefore in describing the biological behavior of ovarian tumors it might be useful to identify the cases with p53 protein overexpression and the aneuploid status. We propose that the cases with normal p53 protein and diploid DNA do much better than the cases with aneuploid DNA content. The data in Table 23 shows that p53 negativity accompanied by diploid DNA content had a significantly better 5 year survival rate 72% than the p53 positive and aneuploid cases.

Also, the S phase of the cell cycle, p53 negative, and diploid cases were correlated with prognostic information in ovarian carcinomas (Table 23), $p = 0.01$.

The cases which were immunonegative but aneuploid may be false negative by immunostaining. Although p53 may not be obligated to mutate for aneuploidy to occur there may be alternate mechanisms. By doing sequencing of these 15 cases in the future we will be able to confirm the immunoreactivity results (Table 20). Henriksen et al., (1994) have shown the presence of p53 overexpression as an adverse variable related to dissemination of disease and residual disease, poor differentiation, and Ki-67 expression. The antibody used in their study was PAb1801. However, Henriksen et al., (1994) were unable to detect any significant relation of p53 protein expression to DNA ploidy or the S phase fraction.

Furthermore, P53 overexpression was associated with negative or lack of $p16^{INK4A}$ expression. P53 mutations as well as loss of $p16^{INK4A}$ expression has been found in some tumors (Okamato et al., 1994). In ovarian carcinoma cases out of 107 tested, the frequency of individual overexpression in both tumor suppressor genes was nearly equal (52% and 48%). These two genes probably behave independent of each other in the genetic or biochemical mode of action in the etiology of ovarian cancer.

However, there is a possibility of normal $p16^{INK4A}$ expression but mutational inactivation of the p53 gene ($p=0.05$, Table 15). Rather, these results suggest that both of these events of probable inactivation of the tumor suppressor genes and stabilization of protein, detectable by immunoreactivity studies are independent of each other. Therefore, the results suggest that p53 does not control $p16^{INK4A}$ protein product in the G1-S phase of the cell cycle. However, the role of wildtype p53 in the G1 to S phase (as a checkpoint

control) includes transcriptional activation of $p21^{WAF1/CIP1}$ (an inhibitor cyclin dependent kinases) and interaction with the DNA repair and synthetic machinery (PCNA, GADD45 as well as $p21^{WAF1/CIP1}$) or proteins that modulate apoptosis (Bax). Hence mutations in the p53 gene in some tumors would consequently abolish the expression of $p21^{WAF/CIP1}$ (Xiong et al., 1993 and El-Diery et al., 1993), and subsequently the loss of $p16^{INK4A}$ function. It is purely speculative, but most likely the loss of $p16^{INK4A}$ and $p21^{WAF1/CIP1}$ function may be important in the etiology of these tumors. In the current study we have also investigated the association between p53 and $p21^{WAF/CIP1}$ overexpression (discussed in section 5.4.2.2).

5.4.2.4 DCC

Fluorescently labeled primers used in this investigation detected 50% (4/8) of ovarian cancers had allelic loss at this marker D18S35 on chromosome 18q21. Allelic loss (29% - 33%) at chromosome 18q21 occurs most frequently in colon cancer (Cawkwell et al., 1994, and Huang et al., 1993). The frequency of allelic loss detected in the two studies using microsatellite markers and in more recent studies further suggest the association of these markers in stage II and stage III CRCs prognosis (Jen et al., 1994). The LOH results in our investigation in a subset of ovarian cancer cases were higher than the combined data from four studies in the past (Cliby et al., 1993, Osborne and Leech, 1994, Sato et al., 1991, and Yang-Feng et al., 1993) that revealed > 30% of the tumors showed LOH on chromosomes 6, 9, 13q, 17, 18q, 19p, 22q, and xp with LOH at 18q being 30%.

The significance of the 50% LOH on chromosome 18q in predicting outcome in ovarian cancer cases needs to be further clarified in a larger study. There are also reports in the literature indicating the involvement of another tumor suppressor gene DPC at the

18q locus (Schutte et al., 1996). In addition, we also determined if LOH, was associated at DCC gene with p53 overexpression and/or ploidy (Table 29). The results did not show such an association in these cases. P53 overexpression and aneuploid DNA content are markers of adverse prognosis in ovarian cancer in many studies including our study (Loakim-Liossi et al., 1997, Silvestrini et al., 1998, and Henriksen et al., 1992). Moreover, p53 overexpression and abnormal DNA content in ovarian cancer have also been indicated to be involved almost simultaneously in the etiology of this disease.

Residual disease was the most significant clinical marker related to survival in a Cox's regression model involving molecular markers such as P53 overexpression, DNA content and %S phase content, age was not significant ($p=0.2$) (Table 35). Consequently it will be necessary to do a more extensive research using molecular markers, in patients with residual disease, to identify the relationship of the usage of these markers to survival.

Table 35: Independent markers related to relative risk of survival and relapse free survival in ovarian carcinoma patients.

Tumor factors	N[1]	RR[2] of death	95% CI[3]	P value	RR[2] of relapse	95% CI[3]	P value
Stage							0.01
ES	58				1	-	
AS	42				2.95	1.26-6.85	
Residual Disease							
NED	53	1	-	0.007	1	-	0.0001
Minimal	20	4.43	1.58-12.41		3.99	1.67-9.51	
Gross	27	8.03	2.73-23.73		7.32	2.91-18.36	

[1]N: number of cases, [2]RR: relative risk, [3]CI: confidence interval.

5.4.2.5 APC

LOH at D5S346 locus for APC gene was,<30% from combined results of four separate allelotyping studies of ovarian cancer (Cliby et al., 1993, Osborne and Leach, 1994, Sato et al., 1991, and Yang-Feng et al., 1993). In sharp contrast, however the results from the current LOH studies in a subset of ovarian cancer cases shows the frequency of 60% (3/5). The relevance of this finding in ovarian cancer needs to be further investigated since there might be an association with its protein expression or loss of protein function (reviewed in Polakis, 1995). It is speculated that the formation of polyps in mice could be the phenotypic presentation of an in vivo disruption of one APC allele (by ethyl-nitrosourea or homologous recombination) results in the expression of a truncated APC protein in addition to the wild-type protein (Fodde et al., 1994). More recent findings question the significance of such studies, however, as the wild-type allele is also lost in the polyp cells (Moser et al., 1995). As shown in Table 29 it is interesting to note all LOH positive cases had diploid DNA content. However, further studies are required to confirm this finding.

5.4.2.6 MSH2

The Lynch II syndrome or HNPCC is characterized by predisposition to tumors of the stomach, proximal colon, ovary and ureter, hepato-biliary system and renal pelvis (Watson and Lynch, 1993). MSH2 gene mutations are rare in ovarian tumors (Fujita et al., 1995).

MSH2 was the first HNPCC gene to be cloned on chromosome 2P (Fishel et al., 1993, and Leach et al., 1993). Mutation in MSH2 or any of the members of mismatch repair genes hMLHI, hPMSI, and hPMS2 cause MSI. The hMSH2 genes belongs to the

MUT-H-L-S family of genes, primarily involved in correcting any DNA mismatches and unpaired DNA loops that may occur by slipped strand mispairing during the replication of DNA that contain simple repeat sequences.

In the present study, MSI in more than one repeat loci was considered as an RER+ phenotype. The immunoreactivity studies showed no significant difference in survival and RFS between the group expressing MSH2 protein (66%, 70/107) compared with the MSH2 negative category ($p=0.8$) (Table 13). These results indicate lack of expression of the MSH2 protein is favorable in prognosis and further data on alteration of DNA methylation may indicate a role in ovarian cancer drug resistance (Dosch et al., 1998). In this study family history of the patients was not available in all the cases examined. Some ovarian cancers in this study may belong to ovarian cancer families that involve high risk 1) site-specific EOC alone, 2) high-risk of both ovarian and breast cancer, and 3) Lynch II cancer family syndrome.

LOH at (D2S123) chromosome 2p16 and the gene MSH2 were observed in 50% (4/8) of the cases evaluated. Combining results of four separate LOH studies indicates up to 20% LOH at this locus (Cliby et al., 1993, Osborne and Leech, 1994, and Sato et al., 1991, and Yang-Feng et al., 1993). RER+ at this locus is estimated to occur up to 5% (1/19) in ovarian tumors (Han et al., 1993). The MSH2 gene, one of the HNPCC family of genes, is responsible for RER+ tumors. Although the HNPCC, DNA mismatch repair, genes are not classically considered as tumor suppressors, there is little doubt they contribute significantly to cancer development in some organs. These group of genes do meet several of the criteria used to define tumor suppressor genes including 1) early onset of disease, 2) LOH does occur in tumors from HNPCC patients, in the case of MLH1 at

least it is the wild-type allele that is lost (Hemminki et al., 1994), and 3) somatic mutations have been detected in nearly 10% of sporadic colorectal tumors (Borresen et al., 1995). There was no significant association between LOH at the MSH2 locus and p53 overexpression or with aneuploid DNA content in a subset of ovarian cancer cases (Table 29).

5.4.2.7 HPC1

HPC1 gene was investigated as a part of this study due to the availability of primers in our laboratory for understanding familial colon cancer risks. The current study shows the LOH results at this locus are probably a non-random event, however the data in the literature shows only upto ~20% LOH at chromosome 1q (reviewed in Godwin et al., 1997). We therefore speculate the presence of another tumor suppressor gene at this locus.

5.4.2.8 MLH1

LOH (D3S1611) at the MLH1 gene on chromosome 3p22 involved 53% (9/17) cases with allelic loss in the primary ovarian cancer cases. Zheng et al., 1991, have observed LOH at 3p locus more frequently (nearly 40%, 6/16) in high grade tumors and absent in low grade lesions.

Whereas other studies using comparative genomic hybridization (CGH) indicate at least 51% increase in DNA copy number at the 3q locus (Arnold et al., 1996). It appears there may be an additional tumor suppressor gene (s) at the 3p22 locus associated with ovarian cancer.

The absence of LOH in 87% (7/8) tumors demonstrates the lack of p53 stabilization and the diploid DNA pattern ($p = 0.2$) in six cases. A recent study by Arzimanglou et al., 1996, indicated a genetic basis of MSI that involving the MLH1 and MSH2 mutation studies which may be different from HNPCC and also distinct MSI patterns may be associated between sporadic and familial ovarian cancers.

5.5 Metastasis

5.5.1 NM23-H1

LOH at the nm23-H1 (chromosome 17q21) was detected in 60% (9/15) cases in the study. The acquisition of invasive and metastatic potential is a key event in cancer progression. Nm23-H1 is a metastasis suppressor gene and its decreased expression in ovarian cancer has been linked to favorable prognosis (Scambia et al., 1996). The LOH assay results from fluorescently labeled microsatellite marker for nm23-H1 gene further suggest nm23-H1 is an important locus that needs to be more extensively studied in AS ovarian cancer patients.

P53 alteration within colorectal cancer, although a late event is an event that precedes the aneuploid DNA content (Carder et al., 1995). This investigation found no association between P53 overexpression and nm23-H1 ($p=0.3$, Table = 29). Furthermore, this lack of association could also be due to the differences in molecular functions of these two genes in controlling cellular proliferation. DNA aneuploidy has been associated with reduced survival in ovarian cancer patients. However, there was no association between abnormal DNA content and nm23-H1 LOH ($p=0.2$), that

consequently raises the possibility in some ovarian tumors with LOH at nm23-H1 and the development of DNA aneuploidy may be an infrequent event.

Chapter 6

6.0 Conclusions

Advanced stage and residual disease were the independent factors related to overall survival and RFS. In advanced ovarian cancer where residual disease is clearly correlated with improving overall survival and RFS it is expected FIGO substaging knowledge would be beneficial in monitoring response or lack of response in a patient. In the present study there was a lack of data on FIGO sub-staging. The studies involving the role of FIGO sub-stages in predicting survival have been investigated in some detail (Partridge et al., 1992).

CA125 serum level measurement was useful in predicting survival and RFS in patients treated with platinum based combination chemotherapy regimen in tumors expressing CA 125. CA125 was not expressed in all mucinous tumors, therefore we suggest the evaluation of CEA in these tumors not inherently expressing CA125. Previously, CEA has been shown to be expressed in 100% mucinous neoplasms (Charpin et al., 1982) and in differential diagnosis between ovarian, and colorectal adenocarcinomas (Yedema et al., 1992). Further studies will be required to investigate CEA role as a serum marker in malignant neoplasms.

One theme that emerges from molecular markers investigated is that in ovarian cancer P53 overexpression and DNA content are prognostic markers. While grade of the cancer is a component that needs to be kept in the three separate categories for analysis purposes. Only after classifying grade into the three categories analysis of prognostic markers would be useful. The results presented here demonstrate that the grade 2 tumors along with P53 overexpression, and grade 2 tumors combined with abnormal DNA

content were predictors of adverse prognosis in ovarian cancer. In this study, % S phase content was relatively more statistically significant in predicting overall survival and RFS. The argument of the use of % S phase content as a prognostic marker is unclear, but several possibilities can be considered.

We also conclude since the grades of the tumor are recognized as distinct phenotypes indicating aggressiveness therefore data analysis may not be valid for predicting outcome if the three grades are combined.

On the basis of the data presented here, ovarian cancer is a complex disease involving mutations. There is also evidence of general genomic instability from MSI and DNA content studies. However, the of % S phase content correlates with outcome events more statistically significantly than DNA content. It would be interesting to determine the association of % S phase content with outcome upon standardized high and low S phase fraction recommendations in a large multicentre study.

The results from molecular markers indicate the commonly observed LOH chromosome arm at a particular loci. It is quite clear form the present study that LOH is a common event but MSI is infrequent. Moreover, there might be a selection of certain tumor suppressor genes for most tumor types, including ovarian tumors (Osborne and Leech, 1994). Molecular and clinical marker multivariate analysis indicated: a)residual disease for survival, b) stage and residual disease for RFS, were independent markers of prognosis.

Chapter 7

7.0 Future Directions

The questions that remain unanswered in this thesis are the following, which if answered, would explain the role of clinical and molecular markers in predicting survival and RFS in ovarian carcinoma. Additionally, the molecular markers would be also useful in defining the genetic pathway in ovarian carcinoma. Recent studies on abnormalities in Cyclin D1 have found the gene to be overexpressed in adenomas of the colon, in other words at a relatively early stage in the process of colon carcinogenesis (Arber et al., 1996).

1. Is appropriate staging being done by surgeons, oncologists and pathologists.
2. Is grading of ovarian tumors and utilization of the traditional grading system in statistical analysis useful in stratifying ovarian cancer patients into high risk and low risk groups for clinical trials?
3. Can we build a model to predict outcome in ovarian cancer patients based on molecular markers from this study! Secondly, is there a selection for certain tumor suppressor gene(s) or oncogenes in ovarian cancer. Obviously, extensive further investigations are required to determine which of these multitude of genes will be useful for detecting abnormalities and then evaluate the prognostic implications.

Depending on the source of the DNA CGH may allow a comprehensive evaluation of all chromosome arms at the same time. The CGH technique enables the identification of regions gain or loss by comparison of DNAs from normal and malignant cells, (Kallionemi et al., 1992). In this technique two fluorochromes are hybridized

simultaneously to metaphase spreads of tumor and normal DNA. The regions of amplification or deletion of DNA sequences are seen as changes in the ratio of the intensities of the two fluorochromes (viz. Fluorescein isothiocyanate and Rhodamine) along the target chromosomes. Thus, CGH enables the detection of random DNA amplification a characteristic of general genomic instability in region of the DNA most likely containing novel oncogenes. Similarly, the detection of the deletions would presumably identify regions that contain tumor suppressor genes. The advantage of CGH is its ability to survey the whole genome in a single hybridization over allelic loss studies that target only one locus at a time. Previously, Butzow et al., 1997, detected DNA copy number changes in ovarian carcinomas to be most frequent in chromosomes 1, 2, 7, 8, 9, 13, and 17.

More recently, novel techniques such as DNA micro arrays has been discovered that can be applied to evaluate the simultaneous expression of thousands of genes and also large scale gene discovery (Ramsay, 1998). The tissue micro array technique would facilitate gene expression and copy number surveys of very large numbers of tumors thus allowing rapid analysis of hundreds of molecular markers in the same set of clinical specimens (Kononen et al., 1998). Large scale genomic analysis coupled with advances in data processing techniques for non-linear based evaluate (neural-network models) may facilitate the identification and usefulness of molecular evaluation of ovarian neoplasms. Therefore, the minute variations in the nucleotides (DNA bases) that can be identified by these novel techniques are of recent vintage and may dictate how drugs may be prescribed by clinicians.

Chapter 8

8.0 References

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Appendix A

Name: _____

Case: _____

SCC : _____

Date of chart review: _____

SHSP# _____

Verification Yes _____ No _____

Hospital _____ P. Access # _____

_____ Access # _____

_____ Access # _____

_____ Access # _____

2nd primary cancer (non-lung, non-skin) Yes _____ No _____

(non-gyn)

Hospital _____ Site: breast _____ bowel _____

Access# _____ other _____

_____Autopsy Yes _____

No _____

Hospital _____

Access # _____

Date of Birth: _____ Age at Diagnosis:_____

Dt Prim Surg: _____ Dt 2nd Look Lap: _____Dt 3rd look lap: _____

Dt of Path Dx: _____ Dt of Death:_____

Cause Ovarian Yes _____ No _____

Other Yes _____ No _____

Unknown _____

Date of Last Follow up: _____

Alive Relapse: Yes _____ No _____ Alive

No Relapse: Yes _____ No _____

Date of 1st Clinical Relapse: _____

Months to Death _____

Months to Relapse _____

Name: _____

Case: _____

SCC : _____

Surgeon: _____

Nature of Surgery/Laparotomy (1st)

bx only _____ BSO _____

TAH _____ omentectomy _____

USO _____ other bx _____

lap & bx_____ U O cyst_____

B O cyst_____

inspection Y N rupture Y N

adhesion Y N ascites Y N

peritoneal cytology +ve -ve unknown

Residual disease

NED _____ μ 1 cm _____ > 1 cm _____ ineval _____

Stage _____ Path _____ Clin _____ unknown ___

Surgical criteric met Yes _____ No _____

(adj only)

Pathology

invasive Yes _____ No _____

LMP Yes _____ No _____

Histo: Serous _____ Papillary _____ Mucinous _____ Undiff _____ Other _____

Other pelvic gyne path: Yes _____ No _____ ineval _____

Grade 1) _____ 2) _____ 3) _____ not done _____

T. S. _____ Inevaluable _____

Bilateral Yes _____ No _____ Unknown _____

Path review Yes _____ No _____

CA-125 Level _____ Date of CA-125 _____

Peritoneal carcinomatosis Y ____ N ____ Ovarian _____ Other _____

Prob Extra ovarian _____

Name: _____

Case: _____

SCC : _____

2nd Look Laparotomy - Nature of Surgery

bx only _____ BSO _____

TAH _____ omentectomy _____

USO _____ other bx _____

lap & bx_____

inspection Y _____ N _____ rupture Y _____ N _____

adhesion Y _____ N _____ ascites Y _____ N _____

peritoneal cytology +ve -ve unknown

Disease Status:

micro _____

macro _____

NED _____

Inevaluable _____

path response _____ CR PR Inevaluable _____

PD SD

Family hx of ovarian Ca (1st deg R) Yes _____ No _____

Family hx of breast Ca (1st deg R) Yes _____ No _____ Family hx of prostate Ca Yes_____ No _____

Family hx of other Ca Yes _____ No _____

(non br.ca) _____

(non ovary) _____

(non skin) _____

Dx of here/fam ovarian/br Ca (HBOC) Yes _____ No _____

Dx of here site specific ovarian (BSSO) Yes _____ No _____

Dx of Here colorectal + endo Ca + _____

ovary ca (Lynch II syndrome) Yes _____ No _____

Name: _____

Case: _____

SCC : _____

P.S. (ECOG) 0 1 2 3 4 unknown

HT. _____ WT. _____ M^2 _____

Chemo: Adj (stage I/II NED)_____ Adv _____

Chemo evaluable Yes _____ No _____

Chemo subtype CP CC PA melphalan CTX CAP Other _____

(1° chemo)

Date of 1st chemo _____

Date of last chemo _____

Date last cycle ends _____

Date of treat prog/death/toxicity _____

(time to treatment failure)

	DDP	CTX	Adria	Carbo	Melphalan	Other
Planned cycle dose						
Planned duration (wks)						
Planned # of cycles						
Planned total dose						
Actual total dose						
% dose						
Planned dose intensity						
($mgm/m^2/wk$)						
Actual dose intensity						

Response Y _____ Prog _____ UNK _____ NA _____

Date of Response _____

CCR - Yes _____ No _____ RT - Yes _____ No _____

Name: _____

Case: _____

SCC : _____

Date 2nd line chemo starts _____

Date of treat[2] failure/death/toxicity _____

2nd line Evaluable for Response Y _____ N _____

2nd line response date _____

CCR Y _____ Progress _____

Unknown _____ Partial Response _____

2nd line chemo Y _____ N _____

Carbo _____ DDP _____ Adria _____

CTX _____ Taxol _____ Other _____

Megace _____ PC _____ Melphalan _____

2nd line other Rx NO _____

Hormone _____ RT _____ SC _____

3rd line Rx

Date of 2nd progression _____

Date of 3rd treatment starts _____

Date of treatment[3] failure/death/toxicity _____

Evaluable for R Y _____ N _____

Response Y _____ N _____ Prog _____ Unknown _____

Response date _____

CCR Y _____ N _____

Chemo Y _____ N _____

Chemotype DDP _____

Carbo _____

Taxol _____

Other _____

RT - Yes _____ No _____ SC - Yes _____ No _____