Prognostic Factors in Early Stages (FIGO I–II) of Epithelial Ovarian Carcinoma

BY

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ABSTRACT


From January, 1988, to December, 1993, 113 patients with FIGO stage IA-IIC epithelial ovarian carcinoma were treated with postoperative radiotherapy. The median follow-up period was 74 months. Tumor recurrences were recorded in 33 cases (30%). The cancer-specific survival rate was 72%. Tumor grade was a significant (P = 0.007) and independent prognostic factor in the multivariate analysis. In a smaller series of 106 patients, a number of prognostic factors (age, FIGO stage, histopathological type, and tumor grade) were studied in relation to regulators of apoptosis (p53, bcl-2, and bax) and growth factor receptors (HER-2/neu and EGFR). Immunohistochemical techniques were used. In a separate series of 103 patients, the DNA content (flow cytometry) and p53 status of the tumors were also studied and related to the same clinicopathological factors. P53 was associated with tumor grade (P = 0.007) and survival status (P = 0.046). In a Cox multivariate analysis, tumor grade (P = 0.0006), bax status (P = 0.020), and EGFR status (P = 0.018) were significant and independent prognostic factors. DNA ploidy of the tumors was strongly associated with tumor grade.

From January, 1994, to December, 1998, a series of 109 patients with ovarian carcinomas (FIGO IA-IIC) were treated with postoperative adjuvant chemotherapy. The same prognostic factors were studied in this series. The median follow-up was 48 months and the cancer-specific survival rate was 75%. Twenty-five (25%) tumor recurrences were recorded. The most favorable survival rate was seen in patients with tumors negative for p53 and positive for bcl-2 or bax. In a multivariate analysis, tumor grade (P = 0.014) and p53 status (P = 0.020) were independent prognostic factors.

Clinical, histopathological and biological prognostic factors should be combined in prognostic models to render patient-tailored therapy possible and to define different prognostic groups for future clinical studies of adjuvant therapy in early stage ovarian carcinomas.

Key words: Ovarian cancer, early stages, adjuvant therapy, prognosis, tumor grade, apoptotic regulators, p53, bcl-2, bax, growth factor receptors, HER-2/neu, EGFR, DNA ploidy.
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INTRODUCTION

Epithelial ovarian cancer, which is the most common (85-90%) histological type of ovarian malignancy, is diagnosed in advanced stages (FIGO III-IV) in two thirds of the patients (1). In the early stages (FIGO I-II), there is also a significant number of recurrences, and death from the disease is not uncommon. At the moment, there is no consensus on the optimal treatment of epithelial ovarian carcinoma in its early stages.

Etiology

Most ovarian cancers appear to arise from the ovarian epithelium, a single layer of cells found on the surface of the ovary (2). Several lines of evidence suggest that incessant ovulation plays a role in the development of epithelial ovarian cancer, and although this relationship is well acknowledged, the underlying causative mechanisms remain unclear. The proliferation of epithelial cells required to repair the disrupted ovarian surface after ovulation could contribute to carcinogenesis by increasing the likelihood of mutations due to spontaneous errors in DNA synthesis. It has been suggested that the high levels of progesterone characterizing pregnancy may induce apoptosis in ovarian carcinoma cell lines and apoptosis has been shown to prevent development of several types of cancer in rodents (3,4,5). Familial (inherited) ovarian cancer accounts for only a small amount (5-10%) of the disease and recent advances in molecular biology have identified the oncogenes BRCA1 and BRCA2, which appear to be associated with inherited breast and ovarian cancer (6,7). It has been reported that infertile women who have used infertility drugs have a 2.8 times increased risk of invasive ovarian cancer and this suggests that gonadotropins or sex steroids may influence the biological behavior of the tumor (8).
**Epidemiology**

Epidemiological studies have shown that pregnancy, breast-feeding, and oral contraceptive use, which inhibit ovulation, protect against the development of ovarian cancer (9). Approximately 900 new cases of ovarian cancer are diagnosed annually in Sweden and it is the fifth leading cancer, accounting for 34% of all gynecological cancer in the country. The incidence in Sweden is 19.2 per 100,000 females per year (10) and is comparable to the incidence of 17.8 per 100,000 in northwestern European countries and North America (11). The ovarian cancer incidence increases with age, the median age in Sweden being 63 years at diagnosis (6,10). The lifetime risk of ovarian cancer for women in the industrialized countries is about 2% (12).

**Prognosis**

Early-stage cancers (FIGO I-II), i.e. cancers confined to the pelvis, are important with regard to optimal therapy, because the majority of these tumors are potentially curable (13). Despite seemingly adequate treatment, early-stage ovarian cancer carries a risk of tumor relapse of 30% (14). According to the latest Annual Report, the overall 5-year survival in FIGO stage I was 82% and, in FIGO stage II, 59% (15). Several series reported in the literature confirm survival rates of approximately 70% in selected patients following whole abdominal radiotherapy (16).

**Clinicopathological features**

There is a distinct association between tumor stage, degree of differentiation, and the age of the patient. Early stage tumors are often better differentiated and the patients are significantly younger than those with advanced stage disease (FIGO III-IV) (17,18). Among the
histopathological subgroups, mucinous and endometrioid cancers are diagnosed relatively more often in stage I and serous and anaplastic tumors, more often in stages II-III (19).

**Tumor biology, regulators of apoptosis, growth factor receptors, DNA ploidy and proliferation activity (S-phase fraction) in ovarian cancer**

**Tumor biology**

Epithelial ovarian cancer is a heterogeneous disease and many biological and molecular factors are important for its development and progression, including growth rate, metastatic potential, and chemo- and radiosensitivity (20). The factors and mechanisms include: (1) oncogene overexpression, (2) mutation or loss of antioncogenes, (3) growth factor and cytokine stimulation, and (4) modification of the host environment and host-antitumor response (2).

Research on cancer treatment indicates that cell death induced by radiotherapy and chemotherapy is predominantly due to apoptosis (3,21). Apoptosis means genetically programmed cell death and is the process of single-cell deletion. In normal tissue, apoptosis is considered to play a vital role in maintaining tissue homeostasis in conjunction with mitosis (22). Types of cell damage that initiate apoptosis include: (1) exposure of cells to ionizing radiation, (2) hyperthermia, (3) toxic exposures, (4) viral infections, (5) cytokines, and (6) chemotherapeutic agents.

**Regulators of apoptosis**

Regulators of apoptosis may be cell-specific and the first gene identified as part of the apoptotic process was bcl-2 (21). The bcl-2 gene is located on chromosome 18, and it encodes for the bcl-2-protein (23). The bcl-2 gene product is unique among the proto-oncogenes, being localized on the mitochondrial membranes and interfering with programmed cell death.
independently of its ability to promote cell division (24). Bcl-2 belongs to a still growing family, the members of which are able to form homo- or heterodimers with one another. The most important regulators of apoptosis are found among these dimers (25,26). Two important new members of the bcl-2 family are bax and bcl-x. It is interesting to note that the bcl-2 members are divided into inhibitors of apoptosis, like bcl-2, bcl-xL, and mcl-1, and promoters (accelerators), like bax, bak, bcl-xS, and bad (21,26,27). It has been shown that an apoptotic signal results in death in cells in which 80% of the bax protein occurs as homodimers, suggesting a crucial role of the bax-bcl-2 balance in the regulation of both proliferation and apoptosis (27). Recent studies show that, by binding to the promoter region of bax, wild-type p53 increases bax expression with resultant apoptosis (28).

The p53 gene is located on the short arm of chromosome 17p and it is a tumor suppressor gene. Mutation or loss of p53 has been seen in many cancers (2,28,29). The product of the p53 gene is a nuclear phosphoprotein which is known to act as a transcription factor, and it has been implicated in the regulation of its own promoter (2,28,30). Induction and overexpression of wild-type p53 protein blocks the cell cycle by pausing in the G1 phase and thereby allowing more time for DNA repair before the cell enters the S-phase of the cell cycle. If this repair is not complete, damaged DNA is recycled, which leads to an accumulation of mutations, increased genetic instability, or even cell death. In case of excessive damage to the DNA, p53 switches off the cell cycle to allow extra time for DNA repair. If the cell is unable to repair its DNA, wild-type p53 may trigger cell suicide by apoptosis, whereas mutant p53 lacks this capability. Mutated p53 accumulates in the cell and is readily detected by immunohistochemical techniques because of the increased stability of the mutated form of the protein (28).
**Growth factor receptors**

Epithelial ovarian cancer is a clonal disease associated with activation of receptor tyrosine kinases (cerbB-2/HER-2/neu, mutant EGFR), cytoplasmic kinases (P13, AKT, src), and monomeric G proteins (ras) (31). Cell membrane receptors that bind growth-stimulating peptides are one class of proto-oncogene products that play an important role in transmitting growth stimulatory signals. These receptors consist of an extracellular ligand-binding domain, a membrane spanning region, and a cytoplasmic tyrosine domain (32). Ligand-induced activation of a receptor tyrosine kinase (RTK) leads to increased phosphorylation of intracellular proteins and of the cytoplasmic tail of the kinase. Removal of phosphate groups from phosphorylated tyrosine by RTKs can down-regulate the growth stimulatory activity of these signaling pathways (33). HER-2/neu is a member of the erbB/epidermal growth factor receptor (EGFR) class I family of receptor tyrosine kinases. The type I family of growth factor receptors includes the epidermal growth factor (EGF) receptor and the tyrosine kinases HER-2/neu (cerbB-2), HER-3/neu (cerbB-3), and HER-4/neu (cerbB-4) (34,35). The cerbB-1 (HER-1/neu) gene codes for the EGFR, a potent mitogen for normal epithelial cells (36). Although encoded by different proto-oncogenes, EGFR and HER-2/neu receptor proteins have a high degree of structural homology (37).

The HER-2/neu proto-oncogene is located on the long arm of chromosome 17 and encodes for a 185 kD transmembrane glycoprotein (p185) with 50% homology with EGFR, i.e., a 170 kD transmembrane glycoprotein receptor (37,38,39).

Various EGF-like factors (EGF) also bind to EGFR, including TGF-alpha. EGF is a 6 kD glycoprotein with a structure similar to that of TGF-alpha, and increased levels have been demonstrated in 30% of ovarian cancers (36,40,41). It is possible that HER-2/neu transduces its signal only through interaction with other ligand-activated HER-/neu family members. Ligand activation of EGFR can result in the formation of homodimers as well as heterodimers.
with HER-2/neu or HER-3/neu (34,40,42). Cell surface growth factor receptors provide promising therapeutic targets for antibodies, immunotoxins, and chemical inhibitors of their kinase activity (43).

**DNA ploidy**

DNA ploidy expresses the nuclear content of DNA as measured by cytometry (44). In normal diploid cells, the DNA content, measured as 2c, corresponds to the amount of DNA measured in 2N (e.g. 2 x 23) chromosomes. Consequently, deviations from the normal chromosome numbers, i.e. aneuploidy, can be determined by the deviation from the normal 2c DNA content (45). The DNA index (DI) is defined as the ratio of the mean c-value of the tumor stemline and the mean c-value of the internal controls. Thus, the normal resting G0 (2N/2c) cells have, on the basis of the DNA content, DI = 1.0. The S-phase fraction is defined as the fraction of replicating cells (those cells between G1 and G2 that are doubling the DNA content) and can be estimated mathematically (44).

**Clinical significance of earlier studies of p53, bcl-2, bax, HER-2/neu, EGFR, DNA ploidy and proliferation activity (S-phase fraction) in ovarian cancer**

**P53, bcl-2, and bax**

In a study of 52 cases of early-stage ovarian cancer, in which 29% of the tumors were p53-positive, Kohler et al. (46) found that p53 status was not related to an adverse outcome. In another study, it was found that 54 of 107 cancers (50%) showed distinct nuclear staining consistent with overexpression of p53 in the majority of the malignant cells. Overexpression of p53 was seen significantly more often in aneuploid ovarian cancers, and it was associated with a higher risk of tumor relapse and a poor outcome for these patients (23,47,48). Levesque et al. found that the presence of p53 overexpression in well or moderately well differentiated ovarian cancers was a strong indicator of a poor prognosis and it was
demonstrated, in a study on early-stage ovarian carcinomas, that p53-positivity of the tumors, in combination with a high tumor grade, was associated with an increased tumor recurrence rate (48). Berchuck et al. (47) found that overexpression of p53 was seen with equal frequency in early-stage carcinoma and in advanced disease. A significant correlation between p53-positivity and tumor grade was reported by W-H Wen et al. (49) in a study of 105 patients with epithelial ovarian carcinomas.

Diebold et al. (23) demonstrated, in a study of 118 patients with ovarian cancer in FIGO stages I-IV, that intense bcl-2 expression occurred in 54% of the cases, and it was seen most frequently in endometrioid carcinomas. In a study of 70 patients with ovarian carcinoma (FIGO stages I-IV), Herod et al. (50), using immunohistochemical staining, found bcl-2 expression in 57% of the cases and p53 positivity in 61%. Both p53 and bcl-2 were significant and independent prognostic factors for survival. The outcome was worse in patients whose tumors showed intense p53 staining, whereas expression of bcl-2 was associated with improved survival.

In a study of 44 patients with tumors in FIGO stages I-IV, Yu-Tzu Tai et al. (51) found bax-positivity in 60% of the cases. A correlation between high bax levels and improved clinical outcome after treatment with taxoids was demonstrated, suggesting that an intact apoptotic pathway is an important determinant of chemoresponsiveness in ovarian cancer. There were no significant differences in stage distribution, residual disease status, histopathological subtypes, or grade distribution between the low-bax and the high-bax groups in their study. In a study by Krajewski et al. (52), reduced expression of bax was associated with a poor response to chemotherapy and a shorter survival in women with advanced breast carcinoma.
**HER-2/neu and EGFR**

Amplification and resulting overexpression of the HER-2/neu proto-oncogene is found in 30% of human breast cancers and in 20% of human ovarian cancers (53). Most studies, but not all, indicate that elevated levels of EGFR and HER-2/neu in both breast and ovarian cancers are associated with a poor prognosis (40). Berchuck et al. (54) found in a study on 73 patients with ovarian cancer that positive staining for HER-2/neu was significantly associated with poorer survival. Slamon et al. (55) also found in a study on 72 patients with epithelial ovarian cancer that overexpression of HER-2/neu was associated with poor survival.

Elevated levels of EGFR have been reported in 36-54% of ovarian cancers using EGF-binding assays, and concordance has been demonstrated by immunohistochemistry (36). There is relatively sparse information available on the incidence and clinical significance of coexpression of EGFR and HER-2/neu in ovarian tumors (43). In a study by Meden et al. (56) based on 275 cases, the ovarian tumors were analyzed by IHC for the HER-2/neu-encoded protein, and there was no significant association between the FIGO stages and the expression of HER-2/neu. In that study, there was no association with tumor grade or histopathological subtype but, in the Cox analysis, the HER-2/neu-encoded protein was an independent prognostic factor, suggesting unfavorable biology for that group of tumors. In a study by Berchuck et al. (57), 77% of the ovarian cancers from 87 women stained positively for EGFR. In a univariate analysis, a statistically significant association between EGFR expression and poor survival was recorded. In another study from Göttingen on 266 patients with epithelial ovarian cancer in FIGO stages I-IV, EGFR was detected in 13% using the IHC technique. Overexpression of EGFR in that study was not found to have any significant impact on the survival rate. The EGFR status of epithelial ovarian cancers has been shown to be of prognostic importance in several retrospective studies analyzed by multivariate technique,
while other studies have not been confirmatory. However, it is possible that increased EGFR expression is associated with more aggressive behavior of the tumor (36).

**DNA ploidy and S-phase fraction**

DNA ploidy has been found to be an important prognostic factor in many studies (58,59,60,61). DNA content and proliferation markers, e.g. the S-phase fraction, have been used as prognostic factors in many studies in an attempt to establish more objective measures of tumor aggressiveness (62). In a study on 118 patients in FIGO stages I-IV, Diebold et al. (63), using flow cytometry on paraffin-embedded tissue, found that nondiploidy was statistically significantly associated with tumor grade, but also with the histopathological subtype, Ki67 index, FIGO stage, and postsurgical residual tumor. In a study by Kaern et al. (60) on patients in FIGO stage I, an aneuploid DNA pattern was seen more frequently in stage IC, in poorly differentiated tumors, in tumors of the serous or clear-cell type, and in tumors of patients over 50 years of age. Schueler et al. (64) found that DI > 1.40 could be used to discriminate between high and low aneuploid stemlines. Klemi et al. (65) demonstrated that DI > 1.30 was the most important prognostic factor in a multivariate analysis and that it was more accurate than using diploid and aneuploid designations in determining survival. In a study from Norway (59) on 290 patients in FIGO stage I, DNA ploidy was an important independent prognostic factor for disease-free survival in a multivariate analysis. However, the degree of differentiation of the tumors was the most powerful prognostic factor in that study. None of the 77 patients in the study with well-differentiated and DNA diploid tumors relapsed. In three flow cytometry studies (46,66,67), it was found that the S-phase fraction was an independent predictor of overall survival. Although the S-phase fraction may be an important predictor of survival, there is no consensus on the cutoff values, and the published studies have used different cutoffs for diploid and aneuploid cell populations (68). In a study by Meyer et al. (69) on 133 patients with ovarian cancer (FIGO stages I-IV), survival of
patients with high-grade ovarian carcinomas could not be predicted by S-phase fraction nor by DNA ploidy. In the same study, low proliferation rates were found when S-phase fractions of 39 tumors of low malignant potential (borderline) were compared with the S-phase fractions of high-grade carcinomas.

**Treatment**

**Surgery and staging procedures**

The FIGO staging system from 1986 and later is a surgical staging system (70). Surgery is one of the cornerstones in the primary treatment of ovarian cancer (71). Several authors point out the importance of an adequate surgical staging procedure for patients with tumors in stages I and II (72). For proper staging and appropriate surgery, a generous vertical midline incision, perhaps extending above the umbilicus, is required (70,72). In early-stage disease, the surgical procedure should comprise total abdominal hysterectomy, bilateral salpingo-oophorectomy, peritoneal washings for cytology, appendectomy, infracolic omentectomy, blind biopsies of the pelvic peritoneum, and right and left paracolic gutters. Adhesions close to the primary tumor in the pelvis should also be biopsied and the pelvic and para-aortic lymph nodes should be sampled. Metastatic involvement of the para-aortic lymph nodes may be encountered even if there are no metastases to the pelvic nodes. The lymphatic drainage of the ovaries via the infundibulopelvic ligaments is regarded as the main pathway.

About 30% of patients with inadequate primary surgical staging are found to have more advanced disease when restaged properly (70,72,73,74,75,76). In presumed stage I disease, occult metastases are found in the omentum in 5% of the cases and removal of the appendix may reveal occult metastases in 4% of the cases (77,78).

Ascites associated with early ovarian cancer contains malignant cells according to cytology in less than 50% of cases. On the other hand, cytology of peritoneal washings in cases with no sign of ascites shows malignant cells in more than 20% of the specimens. Multiple blind and
random biopsies from the pelvic and abdominal peritoneum will detect occult peritoneal metastases in 5-10% of the patients. Some 5-20% of patients with apparent stage I or II ovarian cancer will have metastases to the retroperitoneal lymph nodes (79,80,81,82).

Schueler et al. found that, in 13 out of 45 patients (29%), the restaging laparotomy resulted in upstaging, with 54% of the tumors being finally allotted to FIGO stage III. The complication rate in secondary staging procedures appeared to be significantly higher than in primary procedures, viz. 77% versus 23%. Upstaging was significantly correlated with the serous histological subtype, but not with age or the histological tumor grade (72).

In a study of early ovarian cancer, lymph node metastases were found in 32 out of 242 patients (13%). The incidence of positive nodes was nearly the same among the patients with tumor stage IA, IB, or IC. The significantly highest incidence of metastases (25%) was found in the group of serous adenocarcinomas when compared with other histological subtypes (83).

Complete surgical removal of all macroscopic disease in early ovarian cancer is not synonymous with tumor cure, since a significant proportion of the patients will eventually die of their disease despite seemingly appropriate surgery (84).

There is a lack of consensus around the world regarding the postoperative management of the early-stage, optimally debulked ovarian cancer (53). Patients who are thought to have early-stage disease on the basis of inadequate staging procedures should either undergo a restaging laparotomy or receive postoperative adjuvant therapy due to the possibility of occult residual disease (1,85).

**Adjuvant postoperative therapy**

There appears to be a subgroup of patients suffering from epithelial ovarian cancer who have a favorable prognosis and who do not require any further treatment after primary surgery. An adequate identification of this subgroup would be beneficial in the planning of further treatment and the follow-up after primary laparotomy (86). The currently recommended
therapy for patients with epithelial ovarian cancers is related to stage. Appropriately staged patients with stage IA, grade 1 tumors require no further treatment, but the remaining stage I patients may be candidates for an abbreviated chemotherapy schedule. Stage II-IV patients are typically treated with 6-9 cycles of systemic chemotherapy following optimal cytoreduction (87). Based on data from the GOG (American Gynecologic oncology Group) trials, most authors would agree that tumors in stage IC, grade 3, and all stage II tumors require additional postoperative therapy (1,76).

Abdominopelvic external beam radiotherapy as an adjuvant postoperative treatment after comprehensive surgical staging in completely resected cases of ovarian carcinoma has been employed worldwide for many decades. During the last 10 years, the use of radiotherapy in this setting has decreased and has often been replaced by adjuvant chemotherapy. This change in treatment policy is not based on firm data from clinical trials, but more from extrapolations of data from the treatment of ovarian carcinoma in advanced stages.

Randomized trials indicate that chemotherapy and intraperitoneal radiophosphorus therapy may reduce the frequency of relapses, but they do not increase the overall survival. There is now increasing pressure to adopt paclitaxel and cisplatin or carboplatin as standard chemotherapy also for early-stage disease, based on the apparent improvement in outcome for patients with suboptimally debulked advanced disease, as reported from the GOG #111 study (76).
AIMS OF THE STUDIES

The aims of the studies were to find new prognostic factors in patients with early-stage (FIGO I-II) epithelial ovarian carcinoma. Immunohistochemical techniques were used to detect the protein products of a number of proto-oncogenes and tumor suppressor genes and flow cytometry to evaluate the DNA profile of the tumor cells.

The aims of the first study (I) were to evaluate postoperative whole abdominal and lower abdominal irradiation as adjuvant treatment in a series of early-stage (FIGO IA-IIC) epithelial ovarian carcinomas. Long-term survival data were analyzed for various stages and risk groups and the rates of early and late radiation reactions were recorded. The results were compared with those reported in other studies.

The aims of the second study (II), limited to adjuvant irradiation of FIGO stages I-II epithelial ovarian cancers, were to evaluate the prognostic importance of the protein products of the tumor suppressor gene p53 and the proto-oncogenes bcl-2 and bax, which are known to be important regulators of apoptosis. Immunohistochemical techniques were used to detect the proteins in the tumor cells.

The aims of the third study (III), limited to adjuvant radiation treatment of FIGO stage I-II epithelial ovarian cancers, were to evaluate the potentially new prognostic information gained by analyzing the expression of the cell membrane receptor proteins encoded by HER-2/neu and EGFR using immunohistochemical techniques.

The aims of the fourth study (IV), limited to adjuvant irradiation treatment of FIGO stage I-II epithelial ovarian cancers, were to evaluate the prognostic importance of DNA ploidy status and the S-phase fraction, analyzed by flow cytometry (FCM), and their relationship to the expression of the p53 tumor suppressor gene product. Clinical and histopathological features of the tumors were also analyzed with regard to tumor recurrences and the cancer-specific survival rate.
The aims of the fifth study (V), limited to adjuvant chemotherapy of FIGO stage I-II epithelial ovarian cancers, were to evaluate new prognostic factors, using immuno-histochemical techniques to detect protein products of the tumor suppressor gene p53 and the proto-oncogenes bcl-2 and bax, which are known to be important regulators of apoptosis.
MATERIAL AND METHODS

The studies were conducted at the Department of Gynecological Oncology, Örebro University Hospital. The patients were consecutively recruited from the total population of the Örebro Medical Region (in central Sweden), comprising 820,000 inhabitants.

Study population and design of studies I-IV

During the period January 1, 1988, to December 31, 1993, 171 women with early-stage invasive epithelial ovarian carcinoma were referred to the Department of Gynecological Oncology, Örebro University Hospital. Fifty-eight patients were excluded from the study. Five of these patients with stage IA tumors received no postoperative treatment, but they were followed up with clinical control visits, as were the treated patients. Four of these patients had tumor recurrences during the follow-up period. One patient with a stage IC tumor, and only 26 years of age at the time of the diagnosis, underwent unilateral salpingo-oophorectomy and is still alive with no evidence of disease after 97 months. Two patients were treated with both radiotherapy and chemotherapy and another three had radiotherapy but were excluded from the analysis because of deviations from the standard treatment protocol. Another 48 cases were treated with adjuvant chemotherapy alone, usually four to six courses of cisplatin and doxorubicin in combination. In 24 of these patients, the tumor stage was IC and in 13 cases it was IIC. For the remaining 11 patients, there were one or more contraindications to radiotherapy, e.g. known abdominal adhesions, a history of ileus or subileus, diverticulitis of the sigmoid colon, or repeated abdominal surgery.

The remaining 113 patients, all in FIGO stages IA-IIC, were treated using a standardized adjuvant radiotherapy protocol and were included in the analyses of these studies. The median age of the patients was 62 years (range 26-82 years). Sixty patients (53%) had a history of
previous abdominal surgery, and 86 patients (76%) had a history of one or more concurrent diseases.

**Surgery and staging**

The standard primary surgery consisted of total abdominal hysterectomy and bilateral salpingo-oophorectomy, omentectomy, appendectomy, multiple peritoneal biopsies, and cytology of ascitic fluid or peritoneal washings. Surgery was performed at five different departments of gynecology and obstetrics in the Örebro Medical Region. All the patients were referred to the Department of Gynecological Oncology 4 to 6 weeks after the primary surgery for final staging and classification of the tumor and treatment planning. All patients underwent a gynecological examination under anesthesia. The histopathological specimens were reviewed and, if necessary, reclassified at the Department of Pathology, Örebro University Hospital. The primary evaluations of the histopathological specimens were done at three different departments of pathology serving the five referring gynecological departments. The FIGO staging system for epithelial ovarian carcinoma from 1986 was used in our series of patients in the year 1988, but from January 1, 1989 the FIGO 1988 (88) system was used in our series of patients. FIGO stage I and II tumors were included and designated early-stage ovarian carcinomas. In contrast to many other series of early ovarian carcinomas, stage IIB and IIC were also included in our series. Thus, all tumors supposed to be limited to the ovaries and the pelvis without spread to the upper abdomen or the lymph nodes were included in this series.

**Postoperative adjuvant therapy and follow-up**

The routine postoperative adjuvant therapy for early-stage ovarian carcinoma confined to the pelvis during this period was irradiation of the lower abdomen and the pelvis. AP fields were
used and treatment was given daily, 5 days a week, comprising 23 fractions. The dose per fraction was 1.7 Gy and it was defined as a midplane dose. In cases with potential tumor spread outside the pelvis, mostly in stages IC or IIC (ascitic fluid, rupture of the tumor capsule) or with a residual tumor (stage II), the whole abdominal cavity was irradiated. AP fields were used and the upper border was set 1 cm above the domes of the diaphragm on expiration. The lower border was set just below the obturator foramina and the lateral borders well beyond the anterior iliac spinae. Both fields were treated daily. The midplane dose was 1.0 Gy per fraction, and 20 fractions were given during 4 weeks. No shielding was employed. As a boost, the lower part of the abdominal cavity (upper border at the disk between L3 and L4) and the pelvis were given another 20.4 Gy with the same fractionation as described above for the lower abdominopelvic fields. The external beam irradiation was given with 18-MV linear accelerators. Parallel with, and in addition to, the external beam therapy, intracavitary vaginal irradiation was given using a high-dose-rate (HDR) afterloading technique (\(^{60}\text{Co}\) or \(^{192}\text{Ir}\)). The total dose was 12.0 Gy, given in 2 fractions and specified at a depth of 5 mm below the surface of the vaginal mucosa with an interval of one week. The upper two thirds of the vagina was treated using a perspex vaginal obturator (diameter 20-30 mm).

The patients were followed up every 3-4 months during the first 3 years, and then every 6 months up to 5 years at the Department of Gynecological Oncology. Between 5 and 10 years, the patients were followed up once a year at the referring department. One patient emigrated to Italy and was lost to follow-up. In most cases of recurrence, the patients were treated with platinum-containing chemotherapy.

**Patient series in studies II, III, and IV**

Of the 113 patients, 106 had their tumors analyzed by immunohistochemical techniques to study the prognostic importance of regulatory factors in the apoptotic process as well as
growth factor receptors. In seven cases, it was not technically possible to perform these analyses. The immunohistochemical analyses were performed at the Department of Pathology, Medical Center Hospital, in Karlstad.

The histopathological specimens were first evaluated at three different departments of pathology (Örebro, Karlstad, and Eskilstuna). All specimens were then reviewed at the Department of Pathology in Örebro and classified according to the WHO criteria for ovarian tumors (Scully et al.) (89). Forty-four of the ovarian carcinomas were of the serous papillary type (42%), 21 of the mucinous type (20%), 25 of the endometrioid type (24%), and 16 cases were of the clear-cell type (15%). No ovarian carcinomas in this series were classified as anaplastic. The tumors were staged on the basis of the FIGO criteria: stages IA-IB, n = 57 (54%); stage IC, n = 31 (29%); stages IIA-IIIB, n = 10 (9%); and stage IIC, n = 8 (8%). The degree of differentiation was determined according to Henson (90). The distribution was as follows: Grade 1, n = 38 (38%); grade 2, n = 45 (45%); and grade 3, n = 16 (16%).

**Clinical response and follow-up**

Primary cure was defined as no palpable tumor in the pelvis or abdomen at the first clinical visit three months after the date of the completed radiotherapy. Clinical data were taken from the patient records and the follow-up data were obtained from clinical registers. The median follow-up time was 87 months and the range was between 57 and 125 months. One patient was lost to follow-up.

**Patient series and design of study V**

During the period January 1, 1994, to December 31, 1998, a series of 113 women with early stage (FIGO stages IA-IIIC) epithelial ovarian carcinoma were referred to the Department of Gynecological Oncology, Örebro University Hospital. The age of the patients ranged from 23 to 88 years, with a mean of 61 years. The primary surgery was performed at five different
departments of gynecology and obstetrics in the Örebro Medical Region, and the patients were then referred to the Department of Gynecological Oncology 4-6 weeks later for final FIGO staging, classification, and treatment planning.

**Surgery and staging**

The standard surgical procedure was: total abdominal hysterectomy, bilateral salpingo-oophorectomy, infracolic omentectomy, appendectomy, multiple peritoneal biopsies, and cytology of peritoneal washings. Pelvic node sampling was also included in the standard surgical procedures in one of the referring departments (Karlstad). The staging procedure was not complete in 18 patients, mostly in cases where carcinoma was not clinically suspected at the primary surgery.

Total abdominal hysterectomy and bilateral salpingo-oophorectomy were done in 102 cases, bilateral salpingo-oophorectomy alone in 7 cases, and unilateral salpingo-oophorectomy in 4 cases. For staging quality, infracolic omentectomy was performed in 86 cases, pelvic node sampling in 20 cases, appendectomy in 48 cases, and blind biopsies of the pelvic peritoneum or adhesions near the primary tumor in 48 cases. Ascites was recorded in 43 cases at primary surgery. The ascitic fluid was negative for tumor cells in 36 cases and positive in 7 in the cytological analysis. In 70 cases without ascites at primary surgery, the cytological analysis of peritoneal washings demonstrated tumor cells in 4 cases. Rupture of the capsule of the tumor was recorded in 51 out of 113 cases (45%). The rupture was spontaneous in 7 cases (6%) and secondary to the surgical procedure in 44 cases (39%).
**Postoperative adjuvant therapy**

In the complete series of 113 patients, 103 were treated according to a standardized adjuvant chemotherapy protocol (cisplatin 50 mg/m² and cyclophosphamide 500 mg/m² as a combination in four courses given every four weeks). The remaining 10 patients were treated with other combinations of adjuvant chemotherapy. Dose reduction was necessary in 10 patients, and the most frequent side effects were nausea, vomiting, and fatigue, which occurred in 67 out of 113 cases (59%), myelosuppression in 20 cases (18%), neuropathy in 3 cases (2%), nephropathy in one case, and cardiotoxicity in one case. No side effects were registered in 21 women.

**Patient series in study of immunohistochemistry (study V)**

Of 113 patients, 109 had their tumors analyzed by immunohistochemistry to study the prognostic importance of some regulatory agents of the apoptotic process. In four cases, it was not technically possible to perform the analyses. The histopathological specimens were evaluated primarily at three departments of pathology (Örebro, Karlstad, Eskilstuna). All specimens were then reviewed and classified according to the WHO criteria for ovarian tumors (89) at the Department of Pathology in Örebro. The immunohistochemical analyses were performed at the Department of Pathology, Medical Center Central Hospital, in Karlstad.

Thirty-four of the ovarian carcinomas were of the serous papillary type (31%), 28 of the mucinous type (26%), 31 of the endometrioid type (28%), and 12 cases were of the clear-cell type (11%). Four ovarian carcinomas in this series were classified as anaplastic (4%).

The 109 tumors were staged according to the FIGO criteria: stages IA-IB, n = 42 (39%); stage IC, n = 43 (40%); stages IIA-IIB, n = 8 (7%); and stage IIC, n = 16 (15%). The degree
of differentiation was determined according to Henson (90). The distribution was as follows:
grade 1, n = 29 (28%); grade 2, n = 38 (35%); and grade 3, n = 36 (35%).

**Clinical response and follow-up**

A primary cure was defined as no palpable tumor in the pelvis or abdomen at the first clinical
visit three months after the date of the completed chemotherapy. Clinical data were taken from the patient records, and the follow-up data were obtained from clinical registers.
The median follow-up time was 48 months and the range was between 15 and 80 months.
The patients were followed up at the Department of Gynecological Oncology every 3-4 months during the first 3 years, and then every 6 months up to 5 years. From 5 to 10 years, the patients were followed up once a year at the referring department. In most cases of recurrences, the patients were treated with taxoid-containing chemotherapy.

**Sampling and preservation of ovarian cancer tissue (studies II-V)**

Tissue samples of the ovarian cancers were obtained at the primary surgery and before the start of the adjuvant radiation or chemotherapy. Specimens for immunohistochemical staining were obtained from the paraffin blocks containing the embedded tissue removed from the tumor at primary surgery. The specimens were stained with hematoxylin and eosin and were then reviewed, classified, and graded by the same pathologist.

**Immunohistochemistry in studies II-V**

Immunohistochemical staining was performed on formalin-fixed and paraffin-embedded tissue using the ABC (avidin-biotin-peroxidase complex) method (Hsu) (91), which is a simple and sensitive method for localizing antigens in formalin-fixed tissues. Sections of 5-µ thickness from at least one representative block from each tumor were deparaffinized in xylene (2 x 10 minutes) and then rehydrated with graded alcohol. The slides were then placed
in a jar containing 250 ml of citrate buffer, pH 6.0 (for p53 and bcl-2) or EDTA, pH 8.0 (for bax) and were pretreated (3.5 minutes) with microwaves in a standard Philips-Whirlpool microwave oven (750W). After the microwave treatment, the slides were cooled slowly for 20 minutes.

**Immunohistochemistry (p53, bcl-2, bax) in studies II, IV, and V**

The immunohistochemical staining for p53 and bax was done in a Ventana 320 ES automated immunostainer according to the manufacturer’s instructions. The endogenous peroxidase activity was blocked with an inhibitor solution (4 minutes at 37°C). The slides were then incubated with the primary antibody for 28 minutes at 37°C and thereafter incubated with a secondary biotinylated antibody (8 minutes at 37°C) and with avidin horse radish peroxidase (HRP) for 8 minutes. The antigen-antibody reaction was visualized by DAB (3.3 diaminobenzidine-tetrahydrochloride, 5%) as a chromogen (8 minutes at 37°C). Copper was used to enhance the stain (4 minutes at 37°C). The sections were then counterstained with Mayer hematoxylin and were then dehydrated, cleared, and mounted with Monex R.

The immunohistochemical staining for bcl-2 was done in the DAKO TechMate™ Horizon immunostainer according to the manufacturer’s instructions. The slides were rinsed in buffer 1, 2, and 3 (DAKO, Copenhagen, Denmark) and tap water and were incubated with anti-bcl-2, followed by a biotinylated secondary antibody. Endogenous peroxidase was blocked with a peroxidase-blocking solution (3% H₂O₂). The slides were then incubated with streptavidin peroxidase and mixed with DAB as a chromogen. They were counterstained and processed as described above.

The antibodies used in the study were the following: DO-7 (for p53), mouse/antihuman (mono); bcl-2 oncoprotein clone 124, mouse/antihuman (mono); and bax polyclonal, rabbit/antihuman (poly), all from the DAKO manufacturer.
**Interpretation of immunohistochemical staining in studies II, IV, and V**

The IHC stains were interpreted together by two of the authors (IS and TS). At this evaluation no information was available on the specific diagnosis and prognosis for the individual cases. A semiquantitative analysis (92) was used and the stains were graded as +, ++, and ++++. The stain for p53 was considered to be positive when strong and widespread granular staining of the nuclei of the tumor cells was found. Sections from strongly positive endometrioid carcinomas were used as external controls. The stain for bcl-2 was considered positive in cases where the majority of the tumor cells exhibited strong staining of the cytoplasmic membrane and, in most cases, together with a strong granular perinuclear cytoplasmic stain. Normal lymphoid tissue in which the B-cells of the mantle zones were strongly positive was used as an external control.

Staining for bax was somewhat difficult to interpret since many of the cases showed faint, obviously nonspecific staining of the cytoplasm. However, the cases that were interpreted as positive showed strong granular and punctuate staining of the cytoplasm in most of the tumor cells. Tonsil tissue in which germinal B-cells were strongly decorated with the antibody was used as an external control.

**Immunohistochemistry (HER-2/neu and EGFR) in study III**

The immunohistochemical staining for HER-2/neu was performed in a Ventana 320 ES automated immunostainer according to the manufacturer’s instructions. The endogenous peroxidase activity was blocked with an inhibitor solution (4 minutes, 37 ºC). The slides were incubated with the primary antibody (c-erbB2, rabbit/antihuman (polyclonal) from the DAKO manufacturer) for 28 minutes at 37 ºC and incubated with a secondary biotinylated antibody (8 minutes, 37 ºC) and with avidin horseradish peroxidase (HRP) (8 minutes, 37 ºC). The antigen-antibody reaction was visualized by DAB (3.3 diaminobenzidine-tetrahydrochloride,
5%) as a chromogen (8 minutes, 37 °C). Copper was used to enhance the stain (4 minutes, 37 °C). Sections were then counterstained with Mayer hematoxylin and were dehydrated, cleared, and mounted with Monex R.

The immunohistochemical staining for EGFR was performed in the DAKO TechMate™ Horizon immunostainer according to the manufacturer’s instructions. The slides were rinsed in buffer 1, 2, and 3 (DAKO, Copenhagen, Denmark) and tap water and were incubated with anti-EGFR (EGFR, 113, mouse (monoclonal) from the manufacturer Novocastra), followed by a biotinylated secondary antibody. Endogenous peroxidase was blocked with peroxidase-blocking solution (3% H₂O₂). The slides were then incubated with streptavidin peroxidase and mixed with DAB as a chromogen. The slides were counterstained and processed as described above.

**Interpretation of immunohistochemical staining in study III**

The immunohistochemical stains were interpreted by two of the authors (IS and TS). The interpretation was done without any information about the specific diagnoses and prognoses for each case; thus, the cases were not identified when the interpretation was made. The stain for HER-2/neu was considered positive when at least 10% of the tumor cells exhibited positive staining of the cytoplasmic membrane and, in most cases, also cytoplasmic staining. Staining of the entire cytoplasmic membrane was not required to consider the tumor positive. The intensity of the immunostain varied somewhat from case to case. No background staining was seen. The stain for EGFR was interpreted as positive when at least 10% of the tumor cells showed distinct staining of the cytoplasmic membrane. The stain was often unevenly distributed within the tumor and, in some cases, there was only focal positivity in different areas of the tumor. There was no nonspecific background staining.
**DNA analysis in study IV**

*Sample preparation for nuclear extraction and staining*

The method of Schutte (93) was used for paraffin-embedded tissue. The tumor tissue was microdissected from paraffin blocks under microscopic control. Briefly, sections of 50-µ thickness were dewaxed, rehydrated, and washed. The microsections from tumor tissue were deparaffinated with 5 ml of xylene and the tissue was disintegrated by Wibrafix vortexing followed by incubation for 60 minutes on a rocking table. For rehydration, the sample was treated with 5 ml of ethanol 99.5% and, after mixing and incubation on a rocking table for 5 minutes, it was treated with ethanol in decreasing concentrations. After rehydration of the sample with distilled water, the suspension was treated with 1.8 ml of 0.25% trypsin for enzymatic digestion, mixed, and incubated at 37°C in a waterbath on a rocking table (Techne shaking bath) over night. After disaggregation, the samples were stained for DNA with propidium iodide (PI).

*Flow cytometry technique and interpretation of histograms*

The flow cytometric analyses were performed with a FAC scan (Becton Dickinson, Sunnyvale, CA). The flow cytometer, configured with a 488-nm argon ion laser which increases light collection efficiency for every cell in the run pushed forward by the sheet fluid to the fluorescence detector caused fluorescence of PI at a wavelength of 580 nm. The light power was recorded and measured in a photocell and the content of DNA could be measured. Diploid cell populations in the sample were used as an internal standard as their peak was referred to other diverging peaks. A total of 15,000 events per sample were acquired. The CV (coefficient of variance) was defined as the standard deviation as a percentage of the mean DNA value of the diploid peak. Chicken and trout red blood cells were used as external
standards. A histogram was considered diploid if the peak had a shoulder or was split with DI < 1.10. Nondiploid histograms had a further G1 peak, having DI > 1.10. A nondiploid histogram is usually split into three subgroups: (1) single aneuploid, (2) tetraploid, > 20% in the G2M peak or a separate G2M peak, and (3) multiple aneuploid if there is more than one nondiploid peak. In summary, if there was more than one G1 peak or if DI was > 1.10, the tumor was considered aneuploid. Samples were excluded when CV exceeded 8%. DI is defined as the mean of the relative DNA content of tumor stemline cells in the G0/G1 phase of the cell cycle divided by the mean of the relative DNA content of nonneoplastic diploid control cells in G0/G1. Therefore, DI was calculated as the ratio of the aneuploid to diploid (G0/G1) peaks (94). The S-phase fraction was manually estimated by the rectangle method.

Statistical analyses

Differences in proportions were evaluated by Pearson’s chi-square, and Yates’ chi-square. The t-test was used to compare means of normally distributed continuous variables. The log-rank test (95) was used to compare survival curves (total and cancer-specific) from Kaplan-Meier analyses (96). Univariate and multivariate Cox proportional hazards regression analyses (97) were used to evaluate of prognostic factors with regard to survival rates. A logistic regression analysis was used to evaluate prognostic factors with regard to tumor recurrences (binary data). The Statistica (StatSoft™) statistical package for personal computers was used for the analyses. Statistical significance was regarded as P < 0.05.
RESULTS

Study I (adjuvant radiotherapy in FIGO stages I-II ovarian cancer)

Primary cures were achieved in 110 of the 113 patients (97%). During the follow-up period, 33 tumor recurrences (30%) were recorded in this series of 110 patients. There were 24 recurrences (26%) in FIGO stage-I and 9 recurrences (45%) in FIGO stage-II tumors.

The grade of the tumor was significantly (P = 0.002) associated with tumor recurrence, but the histopathological subtype was not. The distribution of the histopathological subtypes was associated with the FIGO substages, however (P= 0.034). The most obvious observation was that mucinous carcinomas (2c) mostly (75%) belonged to FIGO stage IA. In the series of the 33 patients with recurrent disease, abdominopelvic metastases were most frequent, while combined abdominopelvic and distant metastases and distant metastases alone were less common. Isolated pelvic recurrences were detected in only 2 cases. Distant metastases occurred with the same frequency in the group treated with whole abdominal irradiation (11%) and the one treated with lower abdominopelvic irradiation (9%). The mean time from treatment to recurrence was 26 months (range 6-92 months) in the complete series.

The overall 5-year survival rate was 69%, the cancer-specific survival rate 72%, and the relapse-free survival rate 70% in the complete series. The cancer-specific survival rate was 74% in FIGO stage I and 63% and in FIGO stage II. Tumor grade was highly significantly (P = 0.002) associated with survival, and it was an independent prognostic factor in Cox’s proportional hazard analysis (P = 0.007). In FIGO stage I (n = 90), the grade of the tumor continued to be the only significant (P = 0.002) prognostic factor. In FIGO stage II (n = 23), tumor substage was not significant nor was rupture of the tumor capsule. In this series, clear cell carcinomas was found to have a surprisingly good prognosis, with a cancer-specific 5-year survival rate of 80%. Ovarian carcinoma in FIGO stage IA, grade 1, had the most favorable outcome with 5 and 10-year total survival rates of 92%. The worst outcome was
noted in ovarian carcinomas in FIGO stage IIC with an overall 5-year survival rate of only 38%.

Early radiation reactions of any type or grade were noted in 93% of the cases. In 12 cases (11%), interruption of the radiotherapy was necessary due to the acute reactions. Late radiation reactions of some type were noted in 58% of the patients but, in most cases, they were of low grade, short duration, and of limited clinical significance. The most common side effect was diarrhea. Nausea and vomiting during treatment were more frequent during whole abdominal irradiation (42%) than during lower abdominopelvic irradiation (23%).

**Studies II, III, and IV (studies of immunohistochemistry and DNA analysis)**

**Series of patients in the studies**

In a series of 106 of the 113 patients with early-stage (IA-IIC) ovarian carcinomas treated with postoperative, adjuvant abdominopelvic irradiation, the protein products of the tumor suppressor gene p53 and the proto-oncogenes bcl-2 and bax were evaluated as prognostic factors. The complete series of 106 patients was also split into subgroups according to various combinations of expressions of p53, bcl-2, and bax. The clinicopathological features and survival were evaluated in the different subgroups.

The growth factor receptors HER-2/neu and EGFR were also evaluated as prognostic factors and the complete series of 106 patients was also split into four subgroups according to the expression of HER-2/neu with or without concomitant expression of the EGFR of the tumors.

**P53, bcl-2, and bax**

The results of the immunohistochemical analyses of the p53, bcl-2, and bax proteins were presented and the associations with the clinicopathological features were illustrated. P53 status was significantly associated with the tumor grade (P = 0.007) and with the cancer-specific survival rate (P = 0.046). Patients whose tumors were positive for p53 had a
significantly worse survival rate than patients with p53-negative tumors. Patients with p53-positive tumors also continued to die of their disease after a period of 5 years.

Overexpression of bcl-2 was seen most frequently in serous papillary carcinomas. Positive bcl-2 staining was infrequent in mucinous carcinomas. A subgroup of patients whose tumors were positive for both p53 and bcl-2 were identified and they had a significantly \((P = 0.020)\) worse survival than patients with tumors expressing other combinations of p53 and bcl-2. The most favorable survival rate was seen in the subgroup of patients whose tumors were positive for p53 and negative for bcl-2 after adjuvant radiotherapy (study II). However, after adjuvant chemotherapy, the most favorable survival rate was seen in patients with p53-negative and bcl-2-positive tumors (study V).

The results of the immunohistochemical analyses of bax showed an association with the age of the patients. Patients with bax-positive tumors were younger than patients with bax-negative tumors \((P = 0.012)\). The lowest rate of bax expression was found in mucinous carcinomas \((10\%)\), and the highest rate in clear cell carcinomas \((44\%)\) \((P = 0.012)\). Survival and bax status were associated with each other, and patients with tumors expressing bax seemed to have a better clinical outcome. The association between bax and p53 expressions was examined. The most favorable combination for survival was negative p53 and positive bax staining, and the most unfavorable combination was positive p53 and negative bax staining.

The complete series of evaluable patients was split into four subgroups according to bax expression with or without concomitant bcl-2 expression of the tumors. Patients in the subgroup with tumors showing concomitant positive bax and bcl-2 staining were significantly younger than the patients with tumors negative for bax and positive for bcl-2 \((P = 0.009)\) or negative for both bax and bcl-2 \((P = 0.046)\). The difference in age was linked to the bax status and not to the bcl-2 status. There was a significant difference in the distribution of the
histopathological subtypes in the various bax-bcl-2 subgroups (P = 0.00002). The most striking finding was negative staining for bax (18 out of 20 tumors) and concomitant negative staining for both bax and bcl-2 (16 out of 20 tumors) in tumors of the mucinous type (2c). There was also an association between bax and bcl-2 staining and the grade of the tumor (P = 0.017). Tumors concomitantly positive for bax and bcl-2 showed the most favorable grade distribution. An inverse relationship was seen between bax-positive staining (with or without concomitant positive bcl-2 staining) and p53-positive staining.

Survival analyses in the four subgroups of bax with or without concomitant staining of bcl-2 showed that the most pronounced difference was between the group with bax-positive (with or without concomitant bcl-2-positive) tumors and the subgroup with bax-negative and bcl-2-positive tumors. The difference was not statistically significant, however.

**HER-2/neu and EGFR**

The association of the HER-2/neu expression (positive or negative) and the clinicopathological features was evaluated. There were no differences in the mean age of the patients, FIGO stage, or histopathological subtype distributions of the tumors in the group of patients with HER-2/neu-positive tumors and the group with HER-2/neu-negative tumors. There was no association between the expression of the HER-2/neu protein and tumor grade or the cancer-specific survival rate.

There were no significant associations between expression of the EGFR protein of the tumor and the age of the patients, the FIGO stage, or the grade of the tumor. On the other hand, the cancer-specific survival rate was significantly (P = 0.025) associated with expression of the EGFR. Among the 29 patients who died of their disease, nearly twice as many (52%) had EGFR-positive tumors as among the 77 patients who were still disease-free
and alive (29%). Positive EGFR staining was seen more frequently in serous carcinomas (1c) than in endometrioid (3c) and clear cell (4c) carcinomas ($P = 0.008$).

The complete series of 106 patients was split into four subgroups according to the expression of HER-2/neu with or without concomitant expression of the EGFR of the tumors.

The patients in the subgroup with tumors that were exclusively positive for HER-2/neu tended to be younger than the patients with tumors with concomitant positive staining for HER-2/neu and EGFR. There was a difference in the distribution of the histopathological subtypes in the four subgroups ($P = 0.038$). The most obvious finding was positive staining for EGFR in 21 out of 44 serous carcinomas and positive staining for HER-2/neu in 6 out of 21 mucinous carcinomas. Survival analyses showed a difference in survival rate between the subgroup with EGFR-negative and HER-2/neu-positive tumors and the subgroup with concomitant EGFR-positive and HER-2/neu-positive tumors, but this difference was not significant.

The patients in the subgroups with EGFR-positive tumors (with or without concomitant HER-2/neu positivity) also continued to die of their disease after a period of 5 years.

**DNA analysis**

DNA ploidy could be assessed in 103 of 113 tumors by the flow cytometric technique. Fifty-one tumors (49.5%) were classified as diploid and 52 (50.5%) as aneuploid. Among the aneuploid tumors, 15 (28.9%) were classified as tetraploid. The DNA index (DI) could be calculated in 101 cases and ranged from 1.00 to 3.69. The mean value was 1.36 and the median was 1.10. The S-phase fraction could be measured in 99 tumors (87.6%). The mean S-phase fraction was 13.9% and the median was 11.5%. The S-phase fractions were low (< 5%) in 25 tumors, intermediate (5-14%) in 36 cases, and high (> 14%) in 38 cases. The DNA ploidy status versus important clinicopathological features was evaluated. DNA ploidy was associated with tumor grade ($P = 0.002$). Sixty-nine percent of the poorly differentiated
cancerous were aneuploid compared to 26% of the well-differentiated ones. Twelve out of
15 clear cell carcinomas showed an aneuploid DNA profile compared to 40 out of 88
carcinomas of other histopathological subtypes (P = 0.013). In clear cell carcinomas, a
tetraploid DNA profile was noted in 42% of the aneuploid cases, but in none of the
endometrioid carcinomas. Totally, 33% of the clear cell carcinomas were of the
tetraploid type compared to 11% of the tumors belonging to other subtypes (P = 0.019). The
DNA index and the S-phase fraction of the tumors were associated with the tumor grade (P =
0.005, P = 0.008). The survival status of the patients was not associated with the DNA ploidy,
DNA index, or S-phase fraction of the tumors in this series of patients.

The relationship between DNA status, p53 expression, and tumor grade

The p53 tumor suppressor gene product was evaluated in 106 tumors. The results were
compared with clinicopathological features and the DNA content of the tumors. Tumor grade
was significantly associated with p53 status (P = 0.007). There were also associations
between p53 status and the DNA ploidy, DNA index, and S-phase fraction of the tumors.
Seventy-four percent of the p53-positive tumors were aneuploid and had a DNA index higher
than the median value (1.10). In p53-positive tumors with an evaluable S-phase fraction, 83%
had values greater than the median, compared with only 34% of the p53-negative tumors (P =
0.0001).

Tumor Recurrences

In the series of 106 patients, 29 had recorded recurrences. Twenty recurrences were located in
the pelvis and/or the abdominal cavity and 9 recurrences were recorded as pure distant
metastases or a combination of distant metastases and recurrences in the pelvis and/or
abdomen. There was no association between the location of the tumor recurrences and the
status of p53, bcl-2, bax, HER-2/neu, or EGFR. There was an association between p53 expression of the tumor and the recurrence rate (P = 0.05). There were twice as many recurrences in the group of patients with p53-positive tumors as in the group with p53-negative tumors. The FIGO stage, the histopathological subtype, and the DNA status of the tumors were not statistically significantly associated with the tumor recurrence rate.

**Prognostic factors in multivariate analyses**

In Cox multivariate analyses, different clinicopathological factors were analyzed with cancer-specific survival as the endpoint. The results showed that the grade (P = 0.0006) and bax status of the tumor (P = 0.020) were independent prognostic factors. The age of the patient, histopathological subtype, p53 status, and bcl-2 status were not independent prognostic factors in these analyses. Positive bax staining of the tumor decreased the risk of dying of ovarian cancer by 80%. In a second analysis, the age of the patient, histopathological subtype, tumor grade, HER-2/neu status, and EGFR status of the tumors were analyzed with cancer-specific survival as the endpoint. Tumor grade (P = 0.0017) and EGFR status (P = 0.018) were independent prognostic factors. Age, histopathological subtype and HER-2/neu status were not significant factors. Patients whose tumors were EGFR-positive had a 2.8 (1.2-6.4) times increased risk of dying of their cancer during the period of observation. In a logistic regression analysis, tumor grade, p53 status, and DNA ploidy of the tumors were analyzed with the tumor recurrence rate as the endpoint. Only the tumor grade was then an independent prognostic factor (P = 0.011). The age of the patient, FIGO stage, and histopathological subtype of the tumor did not influence the rate of tumor recurrence.
**Results, study V (adjuvant chemotherapy)**

**Series of patients**

In a series of 109 patients with early stage (IA-IIC) ovarian carcinoma treated with postoperative, adjuvant chemotherapy, the protein products of the tumor suppressor gene p53, and the proto-oncogenes bcl-2 and bax in the tumor tissue were analyzed by immunohistochemistry. The expressions of these proteins were evaluated as prognostic factors. The patients were also split into subgroups according to various combinations of expressions of p53, bcl-2, and bax. The clinicopathological features and the survival rates were evaluated in these subgroups. In the complete series, the 5-year cancer-specific survival rate was 74.5%.

Positive p53 staining was seen most frequently in serous papillary carcinomas and less frequently in clear cell carcinomas. Tumor grade was significantly associated with the p53 status ($P = 0.014$). There was also an association between primary persistent disease and p53 status ($P = 0.016$). In the group of 8 patients who did not achieve a primary cure, 6 had p53-positive carcinomas. The cancer-specific survival rate was associated with the p53 status ($P = 0.007$). Patients whose tumors were positive for p53 had a significantly worse survival rate than patients with p53-negative tumors.

There was an association between the bcl-2 status of the tumor and the histopathological subtype ($P = 0.029$). Overexpression of bcl-2 was seen most frequently in endometrioid carcinomas, but was infrequent in mucinous carcinomas. Tumor grade was also significantly associated with bcl-2 status ($P = 0.034$) associated with bcl-2 status. The bcl-2 status of the tumors did not influence the probability of survival, however.

There were no significant associations between the bax staining of the tumor and the histopathological subtype, tumor grade, or survival status. The lowest rate of bax expression
was found in serous and anaplastic carcinomas and the highest in mucinous and clear cell carcinomas.

In the analysis of the four subgroups according to the p53 and bcl-2 status of the tumors, we found a difference in the distribution of the histopathological subtypes (P = 0.032). The most obvious finding was negative staining for both p53 and bcl-2 (9 out of 12 tumors) in the tumors of the clear cell type. There was also an association between the p53 and bcl-2 staining and the tumor grade (P = 0.011). Tumors concomitantly negative for p53 and bcl-2 showed the most favorable grade distribution. In the subgroup of tumors showing positive staining for p53 and bcl-2, 50% were poorly differentiated. Patients in the subgroup whose tumors showed negative staining for p53 and positive staining for bcl-2 had the most favorable survival rate. The differences in survival rates between the four subgroups were highly significant (P = 0.005). There was also a difference between the four subgroups with regard to primary cure (P = 0.014). Among the 8 patients who did not achieve a primary cure, 6 had tumors belonging to the subgroup that stained positive for p53 and negative for bcl-2. Death due to the disease was most frequent in patients with tumors that stained positively for p53 and negatively for bcl-2.

In the analysis of the four subgroups according to the p53 status and bax expression of the tumors, the FIGO-stage distribution showed significant (P = 0.014) differences. One subgroup of 34 tumors was positive for both p53 and bax and another subgroup of 11 tumors was negative for both p53 and bax. It was noted that most of the tumors that stained negative for p53 and positive for bax belonged to FIGO stages IA-IB, and the same was true for the subgroup of tumors that were both p53-negative and bax-negative. On the other hand, it was noted that most of the tumors in the two subgroups that stained positive for p53 with or without concomitant bax-positivity belonged to FIGO stages IC-IIC.

There was no difference in the distribution of the histopathological subtypes in the four
subgroups, but there was a significant association between p53- and bax-staining and tumor grade \((P = 0.034)\). The most obvious finding was that poorly differentiated tumors often belonged to the subgroup that stained positive for both p53 and bax.

Survival analyses demonstrated highly significant \((P = 0.008)\) differences in cancer-specific survival rates between the four subgroups. The most favorable survival rate was seen in the largest subgroup of patients whose tumors stained negative for p53 and positive for bax. The worst survival rate was seen in the subgroup of patients whose tumors were positive for p53 and negative for bax. Among the 8 patients who did not achieve a primary cure, 6 belonged to the subgroup whose tumors stained positive for both p53 and bax.

The complete series of 108 evaluable tumors was also split into four subgroups according to bcl-2 expression with or without concomitant bax expression. Primary persistent carcinomas were only seen in bcl-2 negative cases. Tumor recurrences and death due to the disease did not differ significantly in the four subgroups. The histopathological subtype distribution varied in the subgroups. Mucinous carcinomas typically belonged to the group staining negative for bcl-2 and positive for bax. The endometrioid carcinomas mainly stained positive for bcl-2 with or without concomitant positive staining for bax. In general, the clear cell carcinomas were bax positive but could be both bcl-2 positive and negative. The most unfavorable tumor grade distribution was seen among bax-negative tumors, irrespective of the concomitant bcl-2 staining properties.

**Tumor recurrences and prognostic factors in multivariate analyses**

The total number of recurrences in the complete series was 25 out of 101 and 60% of these patients died of their disease during the follow-up period. There were no associations between the location of the tumor recurrences and the p53, bcl-2, or bax status. In a univariate analysis, the tumor recurrence rate was significantly associated with the histopathological subtype \((P = 0.041)\). The most striking finding was that only one recurrence was seen in the
25 patients with mucinous carcinomas. The tumor recurrence rate was also significantly (P = 0.007) associated with tumor grade. Only 2 of 25 well-differentiated tumors recurred. Tumor grade and the FIGO substage were the only significant and independent predictive factors with regard to the tumor recurrence rate. Tumor biology, as reflected by the expression of p53, bcl-2, and bax proteins, was nonsignificant in a logistic regression analysis. In a Cox proportional hazard regression analysis, tumor grade and p53 status were the only independent and significant prognostic factors with regard to the cancer-specific survival rate in this series.
SUMMARY OF THE RESULTS

Age was not a prognostic factor in the present studies.

The FIGO stage was not a significant prognostic factor for cancer-specific survival in our series, but it was an independent predictive factor of tumor recurrences (study V).

The histopathological subtype was not a prognostic factor, but an association with FIGO substages was seen. Mucinous carcinomas (2c) mostly (75%) belonged to FIGO stage IA.

Tumor grade was the single most important predictive factor for tumor recurrence and prognostic factor for the cancer-specific survival rate (studies I-V). Tumor grade was associated with p53, bcl-2, and DNA status (DNA ploidy, DI, and S-phase fraction) of the tumors.

P53 positivity of the tumors implied a significantly worse survival rate compared with patients with p53-negative tumors. Patients with p53-positive tumors also continued to die of their disease after a period of 5 years. Positive p53 staining was seen most frequently in serous papillary carcinomas but was infrequent in clear cell carcinomas. P53 status was an independent and statistically significant prognostic factor in multivariate analyses with cancer-specific survival as the endpoint in the series of patients treated with adjuvant chemotherapy.

Bcl-2 overexpression was seen most frequently in serous papillary carcinomas and in endometrioid carcinomas, but it was infrequent in mucinous carcinomas. Bcl-2 status was also
associated with tumor grade. Bcl-2 alone was not an independent prognostic factor for survival, but the information of combined bcl-2 and p53 staining, and combined bcl-2 and bax staining was of prognostic importance.

**Bax** expression in the tumors showed an association with younger age and was found most frequently in clear cell carcinomas. Patients with bax-positive tumors showed better survival than patients with bax-negative tumors. The bax status of the tumors was an independent prognostic factor in the Cox multivariate analysis, where positive staining decreased the risk of dying of the cancer.

**P53 and bcl-2** in combination was a prognostic factor for survival. A subgroup of patients (study II) whose tumors were positive for both p53 and bcl-2 had a significantly worse survival rate than patients with tumors in the other 3 subgroups. In study V, the worst survival rate was found in the subgroup of patients with tumors positive for p53 and negative for bcl-2. There was a difference in the distribution of the histopathological subtypes, and the most striking finding was concomitant negative staining for p53 and bcl-2 in clear cells tumors. There was also an association between p53 and bcl-2 staining and tumor grade.

**P53 and bax** in combination was a prognostic factor for survival (studies II and V). The most favorable combination was negative p53 and positive bax staining, and the most unfavorable combination was positive p53 and negative bax staining. There was a difference between the four subgroups with regard to the FIGO substage and tumor grade. Tumors that stained negative for p53 and positive for bax mostly belonged to FIGO substages IA-IB and poorly differentiated carcinomas mostly belonged to the subgroup that stained positive for both p53 and bax.
**Bax and bcl-2** in combination was not a prognostic factor for survival. The histopathological subtype distribution varied in the four subgroups. The most notable finding was negative staining for bax and bcl-2 in mucinous carcinomas. Tumor grade distribution varied in the four subgroups. Tumors with concomitant positivity for bcl-2 and bax had the most favorable grade distribution. The most unfavorable tumor grade distribution was seen among bax-negative tumors irrespective of the bcl-2 staining properties.

**HER-2/neu** was not an independent prognostic factor. When the complete series was split into four subgroups according to the tumor expression of HER-2/neu with or without concomitant expression of EGFR, a difference in the distribution of the histopathological subtypes was demonstrated.

**EGFR** was an independent prognostic factor, and patients whose tumors were EGFR-positive had an increased risk of dying of their disease during the period of observation. Patients with EGFR-positive tumors also continued to die of their disease after a period of 5 years. Positive EGFR staining was seen more frequently in serous adenocarcinomas (1c) than in the endometrioid (3c) or clear cell (4c) carcinomas.

**HER-2/neu and EGFR** in combination were not a prognostic factor for survival. There was a difference in the distribution of the histopathological subtypes in the four subgroups. The most obvious findings were positive staining for EGFR in serous carcinomas and positive staining for HER-2/neu in mucinous carcinomas.
**DNA status** was not a prognostic factor for survival in the present study, but DNA ploidy was associated with tumor grade. Sixty-nine percent of the poorly differentiated carcinomas were aneuploid compared to 26% of the well-differentiated ones. The DNA index and S-phase fraction of the tumors were also associated with tumor grade.

**DNA and p53 status** in combination were not a prognostic factor. But there was a highly statistically significant association between p53 status and DNA ploidy, DNA index, and the S-phase fraction. Tumor grade was also associated with p53 status and DNA ploidy.
DISCUSSION

Prognostic factors associated with ovarian cancer may be of the clinical, pathological, or biological type. The prognostic factors evaluated in the present thesis were classified in the same way, with age and FIGO stage being regarded as clinical factors, histopathological subtype and tumor grade as pathological factors, and the biomarkers being evaluated as biological factors. The biomarkers can be grouped into five categories: (1) cell growth regulators (p53, bcl-2, and bax), (2) proliferation factors (HER-2/neu and EGFR), (3) nuclear DNA content (ploidy, DNA index, and S-phase fraction), (4) gene products associated with drug resistance (e.g. MDR1), and (5) angiogenic factors (e.g. VEGF) (98). In the present thesis, biomarkers in the first three categories were evaluated as prognostic factors in early stages (FIGO I-II) of epithelial ovarian carcinomas.

Clinical factors

Age was not a significant prognostic factor in the present studies. However, patients with p53-positive tumors tended to be older than those with p53-negative tumors, but the difference was not statistically significant. The same tendency was demonstrated in a study by Levesque (48). It was also noted that patients with bax-positive tumors were significantly younger than patients with bax-negative ones. Some authors (99,100,101) have shown that younger age is a favorable prognostic factor. In our series, a better clinical outcome was seen among patients whose tumors were bax-positive and perhaps the younger age of the patients has also contributed to the good prognosis.

The FIGO substage was not a significant prognostic factor for cancer-specific survival in our series but, on the other hand, only FIGO stage I-II tumors were included. A number of prognostic factors for patients with early stage ovarian cancer are not included in the FIGO staging system, which is based on the findings at primary surgery. The latest FIGO staging system (1988) includes features such as cyst rupture, capsular excrescences, and positive
cytology, without it being clear whether these features are significant and independent prognostic factors. Furthermore, this classification does not take into account the degree of differentiation, which is the most powerful prognostic factor in early stages of ovarian carcinoma (76,102). Another problem is incomplete surgical staging, which causes uncertainty about the spread of the tumor and carries a potential risk of understaging the tumor. In patients with inappropriate initial surgical staging, studies have shown that second surgery results in upstaging of 20-30% of the tumors (80,103). This may explain why the FIGO substages were not of prognostic importance for cancer-specific survival in our series. Understaging of the tumors may also explain why the mortality rate in our series is rather high, despite the fact that all patients received postoperative adjuvant therapy. Today, treatment planning is mainly based on the FIGO staging system despite its limitations, and it could be argued whether or not this system is the best instrument for planning optimal treatment for the individual patient.

Pathological factors

The type of histopathology was not a prognostic factor for cancer-specific survival in the present studies but, in univariate logistic regression analyses, an association with the tumor recurrence rate was found (study V). Most recurrences were seen among patients with serous (38%) and clear cell (36%) carcinomas, compared to only 4% among patients with mucinous carcinomas. Diebold et al. (104) found, in a study from Munich, a 5-year survival rate of 34% for serous carcinomas (all FIGO stages), compared to 62% for mucinous carcinomas and 67% for endometrioid carcinomas. However, it has to be taken into account that endometrioid and mucinous carcinomas are diagnosed relatively often in early FIGO stages, whereas most serous carcinomas are diagnosed in advanced stages. Most studies report a low prognostic value for histological subtype, but serous and undifferentiated carcinomas tend to shorten survival (99,105,106,107). Clear cell carcinomas had a relatively good prognosis with a
cancer-specific 5-year survival of 80% in our series. Many other studies have reported clear cell type to be a very unfavorable prognostic factor when analyzed stage by stage because of its relative insensitivity to chemotherapy (74). It is possible that this can explain the high recurrence rate after adjuvant chemotherapy (study V) compared to adjuvant radiotherapy (studies I-IV).

Tumor grade was the single most important predictive factor for tumor recurrence and the single most important prognostic factor for the cancer-specific survival rate. Thus, we have confirmed the results reported by many other authors (59,71,85,108,109). The purpose of performing multivariate analyses of prognostic factors is to identify significant and independent factors that could be used in treatment planning. At the moment, FIGO stage is used when the treatment plan is drawn up. Sometimes tumor grade is also taken into account, e.g. in stage IA where, according to NIH (National Institutes of Health, USA) recommendations, no further treatment is required for appropriately staged well-differentiated tumors (103,110). Some authors advocate that tumor grade (102), the most powerful prognostic indicator in early stage ovarian cancer, should be used more frequently in therapy planning and that it should also be included in the FIGO staging system. The problem regarding the reproducibility of the tumor grade may affect its prognostic value, however.

Several investigators have shown that tumor grade and histopathological type are not readily reproducible by different as well as the same pathologists (111,112,113,114). In a study on 102 patients with FIGO stage I tumors treated with surgery alone, Brugge et al. (115) evaluated the reproducibility and the prognostic value of histopathological typing and tumor grading between three pathologists. Univariate analyses showed significant differences between grades for all observers. The 5-year survival rate per grade showed considerable variation within and between the observers. Therefore, we need other and more reliable and reproducible factors to tailor an optimal treatment plan for the individual patient. Tumor grade
was associated with both the p53 and the DNA status of the tumors. Poorly differentiated carcinomas tended to be DNA aneuploid and, in addition, often stained positive for the p53 protein.

**Biological factors (p53, bcl-2, and bax)**

P53 was a prognostic factor in our studies indicating a poor prognosis for patients with p53-positive tumors. Furthermore, p53 was associated with tumor grade, the single most important predictive factor for tumor recurrence and the cancer-specific survival rate. The association between a high tumor grade and p53 positivity is in agreement with the findings of Wen et al. (49) and Kupryjanczyk et al. (67). In our studies, there were no significant associations between the FIGO stage and the p53 status of the tumors. On the other hand, our series included only tumors in early (I-II) stages. Berchuck et al. (47) found that overexpression of p53 was seen with equal frequency in early stage carcinoma and in advanced disease. Allan et al. (116) and Geisler et al. (117) found, however, an association between higher p53 expression and advanced tumor stages.

Positive staining for p53 was seen in 22% of the cases in our series. In a study on 347 patients, all with stage I tumors, 50% of the tumors stained positively for p53 and it was concluded that the high frequency of p53 abnormalities indicated that p53 alterations might be an early event in the pathogenesis of ovarian neoplasia (118). The various frequencies of positive p53 staining reported in the literature can be explained by different antibodies used in the immunohistochemical analyses, various definitions and cut-off levels of p53 positivity, and a limited number of cases analyzed or different selections of patients in the studies presented. Positive p53 staining was seen most frequently in serous papillary carcinomas in our series and the same was confirmed by Milner et al. (119) and by Gotlieb and Berek (120). Most clear cell carcinomas stained negatively for p53, and these results are in agreement with those presented by other authors (118,121,122). Therefore, p53 alterations do not seem to
play an important role in the development of clear cell carcinomas. In three recent large retrospective studies of patients with ovarian carcinomas, Diebold et al. (23), Klemi et al. (123), and Reles et al. (124) found that p53 overexpression was associated with the serous histopathological subtype, with high tumor grade (poorly differentiated carcinomas), and poor survival. In two of these studies (123,124), no association was found between p53-positivity and advanced FIGO stages.

P53 status was a significant prognostic factor for survival in a univariate analysis in study II (adjuvant radiotherapy); in study V (adjuvant chemotherapy), p53 status was an independent and prognostic factor also in a multivariate analysis with cancer-specific survival as the endpoint. Our conclusion is that p53 is an important prognostic factor in ovarian carcinoma, even in the early stages. Poorly differentiated carcinomas with positive staining for p53 seem to be of a biologically aggressive type. This should be taken into account in postoperative treatment planning.

Bcl-2 was not a prognostic factor for cancer-specific survival, but the combination of bcl-2 and p53 staining was of prognostic importance for cancer-specific survival, and bcl-2 and bax stainings were associated with the histopathological subtype and tumor grade. Expression of bcl-2 was seen most frequently in serous papillary and endometrioid carcinomas, but it was infrequent in mucinous carcinomas. Diebold et al. (23) demonstrated in a study on 118 patients with ovarian cancer in FIGO stages I-IV that intense bcl-2 expression was associated with a low tumor grade (well-differentiated tumors) and it was seen most frequently in endometrioid carcinomas. Ben-Hur et al. (125) showed in a study that overexpression of bcl-2 was not seen in mucinous ovarian tumors of any grade, whereas it varied between 40 and 60% in serous tumors. The highly dynamic nature of the regulation of the bcl-2 family of genes and the relative importance of the different complexes formed can explain why the determination of bcl-2 expression at one point in time is likely to give limited information
The prognostic importance of bcl-2 seems to depend on its association with other biologically active substances. The association with endometrioid and serous papillary carcinomas indicates a role in apoptosis and tumorigenesis in these histopathological subtypes. Bax status of the tumors was an independent prognostic factor in the patients treated with radiotherapy (study II). Positive bax staining of the tumor decreased the risk of dying of disease by 80%. In the chemotherapy-treated patients (study V), bax alone was not a prognostic factor for the cancer-specific survival rate, but when analyzed in combination with p53 the absence of bax expression was a poor prognostic sign. In a study by Yu-Tzu Tai et al. (51), a correlation was found between high bax expression and improved clinical outcome after treatment with taxoids, suggesting that an intact apoptotic pathway is an important determinant of chemoresponsiveness in ovarian carcinoma. Positive bax expression was seen frequently in clear cell carcinomas in our studies. It may be concluded from our results, as well as from those of others, that absence of bax expression is a poor prognostic factor.

**Comparison of different factors (p53-bcl-2, p53-bax, bcl-2-bax)**

P53 and bcl-2 in combination were a prognostic factor for cancer-specific survival. A subgroup of patients was identified in study V (adjuvant chemotherapy) whose tumors were positive for p53 and negative for bcl-2, and they had a significantly worse survival rate than patients in other subgroups. Patients with tumors showing negative staining for p53 and positive staining for bcl-2 had the best survival rate. In a study from Oslo (127) on 185 patients with tumors in FIGO stage III, the combination of p53 and bcl-2 expression was an independent prognostic factor for survival after chemotherapy. The results of that study were in agreement with our findings. Geisler et al. (128) also found in a study on 103 patients with FIGO stage I-IV tumors, that a combination of p53 and bcl-2 staining was an independent predictive factor of survival. There was a difference in the distributions of the
histopathological subtypes in the four subgroups of combined p53 and bcl-2 staining. The most obvious finding was concomitant negative staining for p53 and bcl-2 in clear cell tumors. There was also an association between the p53 and bcl-2 staining and the tumor grade. Tumors belonging to the subgroup with the most unfavorable survival (p53-positive and bcl-2-negative) were poorly differentiated in 50% of the cases. In the radiotherapy group (study II), different survival rates were also seen in the four subgroups. Surprisingly, patients whose tumors were positive for p53 and negative for bcl-2 had a favorable survival rate but the worst survival was seen in the subgroup of patients whose tumors were positive for both p53 and bcl-2. The type of adjuvant therapy (radiotherapy vs. chemotherapy) may explain these different effects of p53 and bcl-2 on survival. Theoretically, the effects could be explained on the basis of studies by Zaffaroni (129) and Strobel (130) showing that tumors with mutant p53 (p53-positive) which are not delayed in the G1 phase are more susceptible to the lethal effects of ionizing radiation. Cell death after radiotherapy occurs by means of apoptosis and the inhibiting effect of bcl-2 on apoptosis could explain the poor survival after radiotherapy of the patients whose tumors stained positive for both p53 and bcl-2.

P53 and bax in combination were a prognostic factor for survival. The most favorable combination was negative p53 and positive bax staining, and the most unfavorable combination was positive p53 and negative bax staining in both study II and study V. There was a difference between the four subgroups of p53 and bax staining with regard to FIGO stage and tumor grade distributions. Tumors staining negatively for p53 and positively for bax belonged mostly to FIGO stages IA-IB. Poorly differentiated carcinomas frequently belonged to the subgroup of carcinomas that stained positively for both p53 and bax. These findings are in line with the results of a study by Sato et al. (131), where tumor samples from 24 patients before and after chemotherapy (cisplatin, doxorubicin, and cyclophosphamide) were examined for expression of p53, bcl-2, and bax. Bax expression was significantly increased in
tumors with p53-negativity (intact wild-type p53 gene) after chemotherapy, but it did not increase in tumors with p53-positivity (mutant p53 gene). P53-dependent apoptosis seems to be strongly related to chemosensitivity in epithelial ovarian carcinomas. Other recent studies (28) have shown that wild-type p53 increases bax expression with resulting apoptosis by binding to the promoter region of bax. These findings are in agreement with our results and confirm the crucial role of p53 and bax in ovarian cancer.

Bcl-2 and bax in combination were not a prognostic factor for survival. However, the histopathological subtype distributions varied in the four subgroups. The most notable finding was concomitant negative staining for bcl-2 and bax in mucinous carcinomas. The tumor grade distribution also varied in the four subgroups and tumors with concomitant positivity for bcl-2 and bax had the most favorable grade distribution, and the most unfavorable grade distribution was seen in bax-negative tumors irrespective of the bcl-2 staining properties. Lohmann et al. (132) demonstrated an association between short overall and disease-free survival and bcl-2 positivity of epithelial ovarian carcinomas and increased overall and disease-free survival in patients whose tumors stained positively for bax and bcl-x. These studies suggest that bax may act as a classic tumor suppressor protein and the loss of bax function may play a role in both malignant transformations and resistance to chemotherapy (51,133). The results of our studies are in line with these hypotheses.

HER-2/neu was not a prognostic factor by itself. In a study by Meden et al. (56) on 275 cancers in FIGO stages I-IV, there was no association between HER-2/neu and tumor grade or histopathological subtype but, in a Cox analysis, the expression of HER-2/neu-encoded protein was an independent prognostic factor, suggesting an unfavorable biology of that group of tumors (39,53). Rubin et al. (134) showed in a study on 105 patients with advanced tumors that the expression of HER-2/neu was not an important prognostic factor. Thus, the
significance of the HER-2/neu oncogene as a prognostic factor in ovarian cancer remains controversial (135).

The expression of EGFR was an independent prognostic factor, and patients whose tumors were EGFR-positive had an increased risk of dying of their disease during the observation period. Positive EGFR staining was seen more frequently in serous papillary carcinomas than in the endometrioid and clear cell subtypes. Goff et al. (136) found in a study that there was no difference between stages I-II and stages III-IV regarding EGFR expression, but they demonstrated an association with tumor grade, but not with the histopathological subtype. An association between EGFR expression and poor survival was recorded in a study by Berchuck et al. (57). This was in contrast to Meden (39), who, in a study in Göttingen on 266 patients with epithelial ovarian cancer, found that EGFR was not a significant prognostic factor for survival. It is possible that increased EGFR expression is associated with more aggressive tumor behavior, however (36). In our study, EGFR expression was shown to be an important prognostic factor for the cancer-specific survival rate and it was independent of other prognostic factors studied.

HER-2/neu and EGFR in combination were not a prognostic factor for survival in the study. The most obvious finding was positive staining for EGFR in serous carcinomas and positive staining for HER-2/neu in mucinous carcinomas. Coexpression of HER-2/neu and EGFR was found in 12 out of 106 tumors (11%) in our series. Harlozinska et al. (137) detected coexpression in 20 out of 63 cases (32%). No association with the histopathological subtypes was noted, but the coexpression of the two tumor growth factor receptors was observed more frequently in FIGO stages III-IV (35%) than in FIGO stages I-II (24%). The difference was not statistically significant, however. The results of that study may indicate that an increase in EGFR expression might be associated with early stages of ovarian tumorigenesis, and the
enhancement of HER-2/neu reactivity may occur jointly with EGFR activation in the
development and progression of ovarian carcinoma.

The members of the EGFR family, EGFR, HER-2/neu, HER-3/neu, and HER-4/neu, may
interact with each other to form homo- and heterodimers. Several other growth factors are
known to bind and activate members of the EGFR family. Conclusions drawn from in vitro
studies have been that coexpression of EGFR and HER-2/neu favours their transactivation
and leads to a potent mitogen signal (138). These findings contribute to our knowledge of the
importance of the EGFR members in carcinogenesis.

DNA status was not a significant prognostic factor for survival. DNA ploidy was associated
with tumor grade, however. Poorly differentiated carcinomas were aneuploid in 69% of the
cases, compared to 26% of the well-differentiated carcinomas. In a study made in Norway
(59) on 290 patients in FIGO stage I, DNA ploidy was an important and independent
prognostic factor for disease-free survival. However, the degree of differentiation of the
tumors was the most important prognostic factor in that study. None of the 77 well-
differentiated and diploid tumors relapsed. In another prospective study from Norway (139)
on 149 patients with stage I high-risk tumors, DNA ploidy was found to be the most
important prognostic factor for disease-free survival. In a study by Curling et al. (140), the
DNA content was measured by flow cytometry on formalin-fixed and paraffin-embedded
tissue. In FIGO stages IA-IIA, 52% of the tumors were diploid and, in stages IIB-IV, 40% of
the tumors were diploid. Berek et al. reported that 65-90% of the tumors in advanced stages
were aneuploid (44). In our study, 80% of the clear cell carcinomas were aneuploid and 33%
of them tetraploid. In a study by Kaern et al. (60), an aneuploid DNA pattern was seen most
frequently in tumors of the serous or clear cell type. In a study on well-differentiated stage I
tumors, DNA ploidy could not predict tumor recurrence. A DNA index of > 1.40 could be
used to discriminate between high and low aneuploid stemlines and was associated with a
poor clinical outcome (64). In our study, the DNA index was associated with tumor grade. Sixty-four percent of the tumors had DI < 1.4 and 36 percent > 1.4. We could not find any significant difference in survival between these two groups, however. On the other hand, the results of analyses of tumors with DI > 1.10 (median) showed a statistically significant association with the grade of the tumor. The S-phase fraction was also associated with tumor grade. The median value of the S-phase fraction was 11.5%. The results of analyses of tumors with S-phase fractions of > 11.5% and < 11.5% showed a statistically significant association with tumor grade. In a study by Reles et al. (68), a high S-phase fraction (> 14.5%), measured by image cytometry on fresh-frozen tissue, was significantly associated with tumor grade and residual tumor after surgery. It was also a significant predictor of survival in univariate analyses, but not in multivariate analyses. In three flow cytometry studies (46,66,67), it was found that the S-phase fraction was an independent predictor of overall survival.

A combination of DNA and p53-status was not a prognostic factor in our study. There were strong associations between p53-status and DNA ploidy, DNA-index, and the S-phase fraction of the tumors, however. Anttila et al. (141) found a significant association between tumor grade and p53-positivity of the tumors and, in the same study, the p53 status was significantly associated with overall survival in 132 patients with tumors in FIGO stages I-II. Diebold et al. (63) found a significant association between aneuploidy and p53-positivity of the tumors. In their series, 85% of the p53-positive tumors were aneuploid, compared to 74% in our series. Geisler et al. (128) found no significant association between the DNA index and p53-positivity of the tumors. The DNA index, tumor grade, and FIGO stage were associated with each other, however. Elimination of functional p53 leads to loss of control at the G1-phase checkpoint in the cell cycle and thus allows the proliferation of cells with damaged DNA (63). A loss of wild-type p53 function results in unregulated cell division and a lack of
DNA repair ability, which ultimately leads to mutations and aneuploidy (128). These findings explain the association between p53 and DNA status in tumorigenesis. Tumor grade was associated with both p53 status and DNA ploidy in our study. It might be concluded that these three biological factors work together in carcinogenesis in ovarian cancer.

**Histopathological and biological properties of persistent and recurrent carcinomas**

It was observed that the serous histopathological subtype was not seen among the eight persistent carcinomas (stage II) and only in one case was the tumor well differentiated. Six of the 8 carcinomas stained positively for p53 and bax. All of these tumors were bcl-2-negative. The grade of the tumor was associated with tumor recurrences. Recurrent disease was infrequent among patients with mucinous carcinomas after a primary cure (study V). There were no significant differences in the rate of recurrences in the various FIGO substages IA-IIC after adjuvant radiotherapy but, after adjuvant chemotherapy, 19 of the 25 patients (76%) with recurrent disease had tumors in the substage IC or IIC, and only two cases were well differentiated. There were twice as many recurrences in the group of patients with p53-positive tumors (43%) as in those with p53-negative tumors (23%). There were also twice as many recurrences in the group of patients with EGFR-positive tumors (40%) as in those with EGFR-negative tumors (20%) (study III). The expressions of p53, bcl-2, and bax were not associated with tumor recurrences after adjuvant chemotherapy (study V). Patients with EGFR-positive and p53-positive tumors continued to die of their disease after a period of 5 years.
**Relationship between histopathological and biological factors**

Histopathological and molecular studies have provided new insights into the molecular basis of ovarian tumors. According to Diebold (104), the correlation between histological phenotype and patterns of molecular alterations can partly explain the different biological behaviors of the various types of epithelial ovarian neoplasms. In agreement with this theory, our results are presented in a table (Table 1) providing a summary and general survey.

**Adjuvant therapy**

**Radiotherapy**

A primary cure was achieved in 110 of 113 patients (97%). The overall recurrence rate in the complete series was 33 out of 110 cases (30%) and all of these patients died of their disease during the follow-up period. In our series of stage I tumors, 26% of the patients died of their disease. In a series of 115 patients with FIGO stage I tumors, in which surgery was complete both macroscopically and microscopically and in which postoperative external beam radiotherapy was given, Reinfuss et al. (142) found that 24% of the patients died of their disease within 5 years. The 5-year cancer-specific survival rate was 72%. Several series reported in the literature confirm survival rates of approximately 70% in patients treated with whole abdominal radiotherapy (16). In 67% of our cases, the sites of the first relapse was confined to the pelvic-abdominal cavity. Other studies report that the first relapse of the tumor is confined to the abdominal cavity in about 85% of the cases (143).

**Chemotherapy**

A primary cure was achieved in 101 out of the 109 cases (93%). In the complete series, the 5-year cancer-specific survival rate was 75%. In a Scandinavian study (139) on high-risk early stage ovarian carcinomas, single-drug carboplatin was compared with no adjuvant treatment. In the carboplatin group, disease-free survival amounted to 86%. In our series, the total
number of recurrences was 25 out of 101 (25%) and 60% of these patients died of their disease during the follow-up period. In our study, 5 out of 25 recurrences (20%) were located outside the peritoneal cavity. In a study by Hamid et al. (144) on 165 patients with ovarian carcinomas (FIGO stages IC-IV), 17% of the relapses were outside the peritoneal cavity.

**Adjuvant therapy versus observation in early stages of ovarian cancer**

In a study made in England (144), 194 patients with FIGO stage-I tumors were followed-up without adjuvant therapy after adequate surgical staging. The median follow-up period was 54 months, and 50 patients (26%) relapsed with a median period of 16 months after the diagnosis. The overall response rate to first-line treatment with single-agent platinum was only 44%. However, the 5-year survival rate was 94% in stage IA, 92% in IB, and 84% in stage IC. In a Scandinavian study (139) on high-risk early stage ovarian carcinoma, single-drug carboplatin was compared with no adjuvant treatment. After a median follow-up period of 46 months, the disease-specific survival was 86% in the treatment group and 85% in the control group. Two other European studies (ACTION and ICON-1) with a similar design were unable to find any significant difference in the survival of patients with surgically adequately staged carcinomas when chemotherapy was compared with no further treatment. Therefore, chemotherapy alone does not seem to be the final solution of adjuvant therapy in early stage ovarian carcinoma (145).

**Prognostic factors for the selection of patients for adjuvant therapy**

Several prognostic factors have been identified for patients with early stage ovarian cancer, some of them being incorporated in the FIGO staging system, but one of the most important prognostic factors, tumor grade, is not considered in that system. The excellent prognosis for patients with stage IA, grade 1 tumors treated with surgery alone is widely recognized (146), but there is a lack of consensus on the best type of postoperative
therapy for patients with tumors in other substages and grades. As a result, a wide variety of postsurgical therapeutic modalities have been used (74). The conclusion from a multicenter study (102) on 1,545 patients with tumors in stage I was that tumor grade, the most important prognostic factor, should be used in therapy decisions and included in the FIGO classification of stage I ovarian cancer. Tropé et al. (139) have also proposed that DNA ploidy should be included in a new FIGO staging system. The FIGO staging system for ovarian carcinoma is a pure description of tumor spread and localization, and the biological properties of the tumor are not taken into account. Therefore, the FIGO staging system, if maintained, should be combined with pathological and biological prognostic factors in conformity with the results of our studies and others reported in the literature. The patients could be allotted to a subgroup with a favorable prognosis with no need for postoperative treatment and to subgroups with intermediate and poor prognoses (Table 2) where efficient patient-tailored therapy, e.g. gene therapy alone or in combination with chemotherapy, could be evaluated.

**Future developments in adjuvant therapy**

Radiation-induced apoptosis appears to be dependent on the presence of normal wild-type p53 protein (147). A cause-and-effect relationship between the expression of wild-type p53 and G1 arrest, which occurs after irradiation, has been established in laboratory studies. G1 arrest was demonstrated after irradiation following transfection of wild-type p53 genes into cells lacking endogenous p53. The G1 arrest after irradiation was lost following transfection of mutant p53 genes into cells with wild-type endogenous p53 genes (148). Chemotherapy is known to induce cytotoxic effects by inducing damage to a proximal drug target, e.g. DNA or tubulin, which then, secondarily, results in apoptosis (51). DNA binding agents, e.g. cisplatin and cyclophosphamide, and agents stabilizing the microtubuli, e.g. taxoids, induce proximal cell damage. It has been reported that cells functionally deficient in p53 or with elevated
levels of either bcl-2 or bcl-xL are relatively resistant to cytotoxic agents, e.g. platinum analogues (149).

During the last few years, significant attention has been paid to corrective therapy with the wild-type p53 gene. Adenovirus-mediated p53 gene therapy (Adwtp53) for cancer is currently undergoing phase I-II clinical trials in several countries. Theoretically, the introduction of wild-type p53 genes into the cells with nonfunctional p53 protein should enhance their sensitivity to most chemotherapeutic drugs (150). Based on an in vivo study (151), in which human ovarian cancer cells were injected into female athymic nude mice, it was suggested, that combination therapy of Adwtp53 and cisplatin was better than single-agent therapy alone, and increased survival (30-40%) was seen. In a study by Xiang et al. (152), the therapeutic potential of bax was examined by overexpressing bax alone or in combination with chemotherapy by using recombinant bax adenovirus on ovarian cancer cell lines. The levels of bax, bcl-2, and p53 and the frequency of apoptotic cell death were evaluated. Overexpression of bax significantly enhanced chemotherapy-induced cytotoxicity and this study suggested that bax alone or in combination with chemotherapy may augment the efficacy of chemotherapy in the treatment of ovarian cancer.

Cell surface growth factor receptors provide promising therapeutic targets for antibodies, immunotoxins, and chemical inhibitors of their kinase activity. Monoclonal antibodies targeting the extracellular domain of the receptor are already being used for cancer therapy (153). The growth inhibitory effect of the antibody alone is cytostatic, with tumor growth recurring after discontinuation of the antibody administration. Treatment with cisplatin and HER-2/neu (rhuMAb) in relatively close temporal proximity appears to be necessary for the greatest suppression of tumor growth (154). Recently, Herceptin®, a humanized form of anti-HER-2 MAb 4D5 was approved for the treatment of patients with metastatic breast cancers overexpressing the HER-2/neu-encoded protein p185 (155). The observation in clinical
studies that tumors showing overexpression of growth factor receptors are associated with a poor prognosis has stimulated preclinical investigations targeting inhibition of the function of human HER-2/neu and EGFR as new cancer therapies (156).
Table 1.
Histopathological subtypes versus biological factors in studies I-V.

<table>
<thead>
<tr>
<th>Histopathological subtype</th>
<th>p53</th>
<th>bcl-2</th>
<th>bax</th>
<th>HER-2/neu</th>
<th>EGFR</th>
<th>DNA-ploidy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serous (1c)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+</td>
<td>aneuploid/diploid</td>
</tr>
<tr>
<td>Mucinous (2c)</td>
<td>-</td>
<td>-</td>
<td>+/-</td>
<td>+</td>
<td>-</td>
<td>aneuploid/diploid</td>
</tr>
<tr>
<td>Endometrioid (3c)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>aneuploid/diploid</td>
</tr>
<tr>
<td>Clear cell (4c)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+/-</td>
<td>+/-</td>
<td>aneuploid</td>
</tr>
<tr>
<td>Anaplastic (5)</td>
<td>+/-</td>
<td>-</td>
<td>+</td>
<td></td>
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</tbody>
</table>
Table 2.
Proposed prognostic grouping of clinical, histopathological, and biological factors.

Favorable prognostic group
FIGO stage IA
Mucinous subtype (2c)
Tumor grade 1
Bax-positivity
Diploid DNA content

Intermediate prognostic group
FIGO stages IB and IIA-B
Endometrioid and clear cell subtypes
Tumor grade 2
Bcl-2-positivity, HER-2/neu-positivity

Poor prognostic group
FIGO stages IC and IIC
Serous and anaplastic subtypes
Tumor grade 3
P53-positivity
EGFR-positivity
Aneuploid DNA content
CONCLUSIONS

Significant prognostic factors for the cancerspecific survival rate in our studies on early stage ovarian cancers were: tumor grade, p53, bax, and EGFR expressions of the tumors. The prognostic factors studied in this thesis could be classified in two groups with regard to their importance for survival. The first group of the most important prognostic factors encompassed tumor grade, p53, bax, and EGFR. The second group of less important factors comprised age, FIGO stage, histopathological subtype, bcl-2, HER-2/neu, and DNA status. The type of adjuvant therapy may also influence the prognostic value of the biological factors. By combining these factors, three prognostic subgroups of tumors with favorable, intermediate, and poor prognoses were proposed. Since patients with an apparent good prognosis according to the known prognostic factors still succumb to their disease, we need better instruments to predict the clinical outcome. One way would be to construct prognostic models with 2, 3 or more clinical, pathological, and biological variables included. These models might also be of importance for future studies of adjuvant therapy in early stages of ovarian carcinomas.
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