BIOLOGICAL MALIGNANCY GRADING IN EARLY-STAGE OVARIAN CARCINOMA

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The usefulness of adjuvant therapy in early ovarian cancer is a matter of controversy and there is a need for predictive methods to distinguish between low and high risk patients. Specimens from 95 early-stage ovarian cancer patients have been analysed for conventional clinical variables as well as for the biological markers—DNA content, MIB-1, p53, WAF-1—and correlated to survival. Prognostic significance achieved in univariate analysis could be improved by using a score based on several biological markers. Using a score based on DNA content, MIB-1, p53 and WAF-1, a significant predictor could be achieved with the aim of determining the postsurgical therapy. By using this tool, it is hoped that adjuvant therapy can be avoided for one-third of the patients with early-stage ovarian cancer.

Early-stage ovarian cancer, in contrast to advanced stage, has a much better survival rate and more than 70% of patients will survive for 5 years. During recent years postsurgery treatment of early ovarian cancer has been modified by excluding patients with well-differentiated tumours from adjuvant therapy. It is clear that this exclusion criterion seems to be inadequate since even though grade is an important prognostic factor, the prognostic significance is not high enough to identify all low risk patients. A relatively large number of cases with well-differentiated tumours succumb to the disease while patients with poorly differentiated tumours may survive. The question of adjuvant therapy in early ovarian cases has been extensively discussed during the past few years. Since selection of the cases of early ovarian cancer in which adjuvant therapy should be applied is an important issue, we investigated a combination of biological malignancy markers in order to facilitate distinguishing between low and high risk patients.

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Material and Methods

The biological markers MIB-1, p53 and WAF-1 (p21), as well as DNA content, were studied in order to test whether prognostic significance could be improved by using a combination of those markers. Material from 95 tumour specimens in paraffin blocks were analysed from patients in FIGO early stage Ia-IIa treated at the Radiumhemmet, Karolinska Hospital, during the years 1979–1986. Borderline cases were excluded. The histopathological specimens from hospitals in the Stockholm area were brought to the Institute of Pathology at the Karolinska Hospital and several representative pieces of tumour indicated by the pathologist (CS) were investigated. The nuclear DNA content and proliferative activity as estimated by the number of MIB-1 positive cells, p53 overexpression and WAF-1 (p21) were analysed from the histological section. The grading was determined according to the WHO classification.

Patients. Altogether, 95 patient specimens were studied. All the patients had undergone surgery in hospitals in the Stockholm area. In all but two cases both ovaries were removed. In one of these cases surgery was conservative because of the age of the patient, and in the other case dense adhesion caused difficulties in identifying the other ovary. In 70 patients total hysterectomy and bilateral salpingo-oophorectomy were performed. In the remaining 23 patients

only bilateral salpingo-oophorectomy was performed since hysterectomy was not required at the beginning of the study period. Histopathological examination revealed that 30 patients had serous, 20 mucinous, 25 endometrioid, 18 mesonephroid and 2 anaplastic carcinoma.

After surgery, all the patients were referred to Radiumhemmet for further treatment and follow-up. The mean age of the patients was 56.6 years, range 26–86 years. According to the current FIGO classification 35 cases were in stage Ia, 7 cases in stage Ib, 45 cases in stage Ic and 8 in stage IIa. All but 10 of the patients had received postoperative adjuvant treatment, 55 with irradiation and chemotherapy, 25 with chemotherapy alone and 5 with irradiation alone. Radiotherapy was given to the lower abdominal field with a dosage of 40 Gy. Until 1982 chemotherapy consisted of melphalan alone, during the period 1982–1985 a combination of melphalan and doxorubicin was given and during 1986 a cisplatinum-based combination therapy was introduced.

Nuclear DNA content. DNA content analysis was performed in 8 μm thick histological sections by means of image cytometry. The tissue preparations were Feulgen-stained and the DNA measured according to previously described methods (1). The DNA histograms obtained by image cytometry were interpreted according to a modified subjective method. The original method was used for the classification of DNA profiles obtained in fine-needle aspiration specimens from breast tumours (2). DNA histograms characterized by a single peak in the 'diploid' or 'near-diploid' region (1.5c–2.5c) were classified as diploid (Subgroup 1). The total number of cells with DNA values exceeding the 'diploid' region (>2.5c) was less than 10%. DNA profiles with a stemline outside the diploid region and distinctly scattered DNA values exceeding the tetraploid region (3.5c–4.5c) were classified as aneuploid (Subgroup 2).

MIB-1 (Ki-67). Immunohistochemical staining was performed using an avidin-biotin-peroxidase complex technique. Briefly, 4 μm sections were deparaffinized and rehydrated. Then the sections were microwave-treated for 10 min at 750 W and 15 min at 425 W in a 0.01 M citrate solution, pH 6.0. After cooling for 15 min, the sections were rinsed in cold water and placed in TRIS buffered saline (TBS) for 5 min. The sections were incubated with MIB-1 antibody (Immunotech, France) for 60 min at room temperature, then rinsed with TBS and thereafter incubated for 30 min with rabbit-anti-mouse-antibody (E 354, Dako, Denmark) diluted 1:200. After rinsing with TBS, the sections were incubated for 30 min with ABC-complex (Dako 377, Denmark) diluted 1:100 in phosphate buffered saline. The slides were subsequently rinsed with TBS and incubated with a peroxidate solution (diaminobenzidine, DAB D5905, Sigma Steinheim, Germany) for 5–10 min. The sections were rinsed with distilled water, counter-stained with hematoxylin and mounted. Stained and unstained nuclei were counted and the percentages of stained nuclei were calculated. In each specimen a minimum of 5 000 cells were scored. Cases with less than 20% MIB-1 positive tumour cells were regarded as of low proliferation (Subgroup 1), those with 20–40% MIB-1 positive cells were intermediately proliferative (Subgroup 2) and those with more than 40% of the tumour cell population stained were judged as highly proliferative (Subgroup 3).

p53 and WAF-1/p21. Expression of p53 was analysed with help of the DO1 monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, USA). Deparaffinized sections, 4 μm thick, were rehydrated and endogenous peroxidase activity was suppressed with 0.5% H_2O_2 for 30 min. DO1 was used at 1:100 dilution. The sections were heated in citrate buffer, pH 6.0, for 5 min at 700 W in a microwave oven in order to intensify the immunohistochemical result. Primary antisera were applied overnight at +4°C to +8°C and the site of the antigen-antibody binding was visualized according to the Avidin-Biotin-peroxidase Complex method (ABC Elite, Vector Laboratories, Burlingame, CA, USA), following the manufacturer's instructions. Diaminobenzidine (DAB) was used as chromogen, and the sections were counterstained with Mayer's hematoxylin.

For staining with WAF-1 (p21) antibody (Oncogene Science, USA), 4 μm thick sections were treated as with the DO1 antibody, but were heated twice for 5 min in the microwave oven.

Only those cells with a distinct staining confined to the nuclei were regarded as p53 or WAF-1 positive. The results of the immunohistochemical p53 and WAF-1 stainings were judged by three investigators, first independently, and then in a joint session. In each specimen, at least 5 000 tumour cells were screened. For p53, assessment of the immunohistochemical staining was from 0 to +++ as follows: 0 = no cells stained (Subgroup 1); + = less than 10% tumour cell nuclei stained (Subgroup 2; ++ = 10–50% tumour cells nuclei stained (Subgroup 3); +++ = at least 50% tumour cells nuclei stained (Subgroup 4).

For WAF-1, the immunohistochemical staining was interpreted as follows: >20% immunoreactive cells = +++ (Subgroup 1), >5% <20% stained cells = ++ (Subgroup 2), <5% stained cells = + (Subgroup 3), no cells stained = 0 (Subgroup 4).

Statistics. Survival was analysed with the logrank test and Cox's regression. Each independent variable (predictor of survival) had two or more categories. From the univariate survival analysis, results were given (in connection with the logrank test) for all four markers. For each patient the score was computed by adding the results of the observed and expected deaths for all four markers. The observed scores for the patients ranged from 1.9 to 6.1. The patient material was then divided into three approximately equally large groups, with respect to the scored values: 1.9–3.0, 3.1–3.9 and 4.0–6.1.

Results

Clinicopathological factors. The 5-year survival rate for all patients was 79% and the 10-year survival rate 72%. There was a statistically significant relationship between survival rate and grade with a 94% 5-year survival rate for well-differentiated tumours and 60% for poorly differentiated tumours. The same applied for stage with an 88% 5-year survival for stage IA and 63% for stage IIA. Tumour size and histology were not of prognostic significance. There was a statistically significant change with age giving a better prognosis for patients younger than 44 years of age (94% 5-year survival), intermediate for patients 45–64 years of age (82%) and a poorer prognosis for patients 65 years of age and more (63%). In stage IC there was a significantly poorer prognosis for patients with tumour growth on the surface, ascites and preoperative rupture (59% 5-year survival) compared with patients with punction or rupture during surgery (85%), as we have published previously (3). There was no statistical difference for the different types of postoperative treatment, except that patients not given chemotherapy did better than patients given chemotherapy. This was probably an effect of patient selection, as patients with good prognostic factors—such as stage Ia and well-differentiated tumours—did not receive postoperative adjuvant therapy.

Biological malignancy grading. DNA content. DNA could be measured in specimens from 67 out of a total of 95 patients, of whom 37 were aneuploid and 30 diploid. There was a significant correlation between ploidy and patients' survival ($p < 0.02$) (Fig. 1).

MIB-1 (Ki-67). All patients could be analysed for MIB-1 activity. The univariate analysis showed no statistically significant correlation between proliferative activity and survival, although a numerical trend was found for an association between decreased survival and increased proliferative activity (Fig. 2).

p53. Of the 95 patients, 39 overexpressed p53. There was a numerical trend, but not a significant correlation between survival and more pronounced overexpression of p53 (Fig. 3).

WAF-1/p21. WAF-1 was expressed in 48 of the 95 patients. There was a borderline significant correlation between better survival and greater expression of WAF-1 (0.06) (Fig. 4).

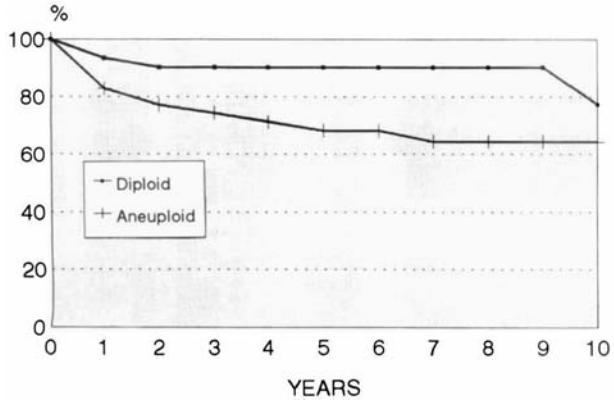

Fig. 1. Correlation between DNA content (diploid, aneuploid) and survival

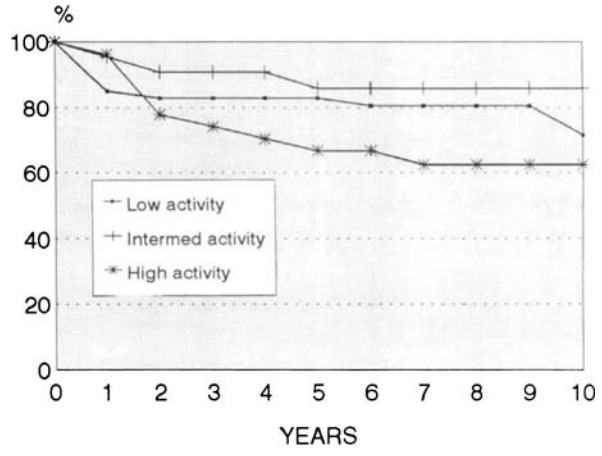

Fig. 2. Correlation between proliferation (low, intermediate and high activity) and survival

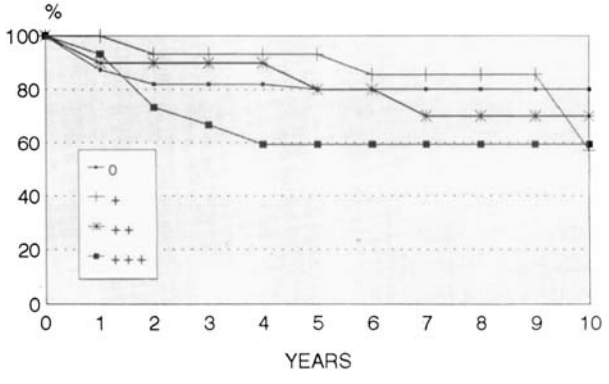

Fig. 3. Correlation between p53 expression (0, +, ++, +++) and survival

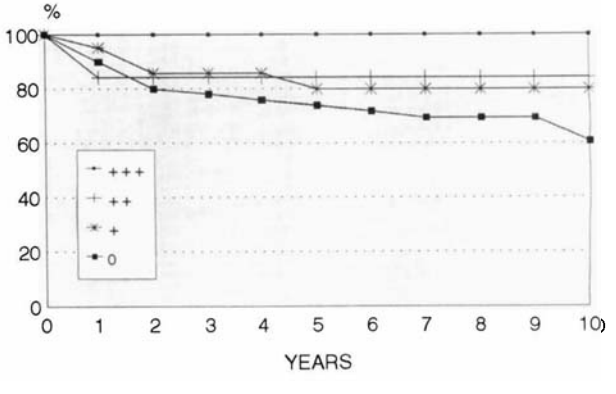

Fig. 4. Correlation between WAF-1 (p21) expression (0, +, ++, +++) and survival

Score. Based on univariate analysis of biological markers, i.e. DNA content, MIB-1, p53 and WAF-1, a score value was established for each patient individually, making it possible to stratify the patients into three risk categories: the lowest risk group had a value of 1.85–3.09, the intermediate risk group had a value of 3.10–3.99 and the highest risk group had a value of 4.00–6.1. Comparison of these three patient risk groups revealed a statistically significant correlation to survival, with $p = 0.01$ (Fig. 5). With Cox's analysis, a combination of four biological markers showed a highly statistically significant relationship to survival ($p = 0.001$). Of 33 patients with a score of 1, three patients died of their disease. Thirty patients had a score of 2 and 8 of them died of the disease. Among 32 patients with a score of 3, 12 died of the disease—all but two were treated very intensively after surgery.

Discussion

Surgery is always the initial treatment in early ovarian cancer. How invasive the procedure should be and whether it should even include lymph node dissection is a matter of controversy. In this study the following question is addressed: Is there a possibility on the basis of biological markers, without performing invasive retroperitoneal surgery, to establish with accurate precision the prognostic factors predicting high risk patients?

Previously established prognostic factors in early ovarian cancer were mostly clinical and histopathological, such as age, grade, tumour size and tumour stage (4–7), but they even included biological factors such as DNA measured by flow cytometry or image cytometry. In the clinical work-up the issue of good prognostic factors in the early stages of ovarian cancer is of greater importance than in the advanced stages. The survival rate for early-stage ovarian cancer is high, almost 80%, but the criteria for exclusion from adjuvant therapy have been difficult to establish. During recent years, several authors have indicated the possibility of excluding patients with well-differentiated tumours from adjuvant therapy and have even advocated that conservative therapy can be considered in young women with stage Ia disease. It is important to identify low and high risk groups in the early stages in order to spare the majority of patients from having to undergo adjuvant treatment.

Even if DNA ploidy in previous studies has been shown to be an independent prognostic factor (8–10), some authors did not find the same correlation and prognostic significance in the early stages (11, 12). From the studies previously reported it can be concluded that there is a considerable number of patients who will relapse despite having diploid tumours (11–13). With other biological markers expressing proliferation genes as well as inactivation of suppressor genes, it may be presumed that the biological malignancy can be established more precisely, with a possible prognostic impact. In several previous studies the p53 overexpression was studied in ovarian cancer, assuming that alteration of the p53 gene is a critical step in human carcinogenesis. Previous studies have shown that the p53 gene was mutated in 30–80% of ovarian carcinomas and that the genetic changes correlated with mutant p53 protein accumulation in tumour tissue. Those studies were mostly carried out in the advanced stages of ovarian cancer and thus they have not been conclusive for the earlier stages (14–19). To our knowledge, the present investigation is the first study in ovarian tumours in which p53 and WAF-1/p21 expression were analysed simultaneously. In several cell types the main regulator of WAF-1/p21 expression is p53 (20). Overexpression of both p53 and WAF-1/p21 indicates the presence of wild type p53, whereas p53 overexpression without WAF-1/p21 expression may indicate mutant type p53.

Only a few studies have been successful in showing a relationship between p53 expression and clinical outcome (21, 22). Levesque et al. (22) have shown that mutant p53 protein accumulation in early stage tumours with low histological grade was associated with an increased risk of cancer relapse. In contrast, in the study presented by Kohler et al. (23) p53 was studied in 52 early-stage ovarian cancers. p53 overexpression was seen in 29% of early-stage cancers and was not associated with an adverse outcome, even though overexpression was seen more frequently in large tumours and in tumours of high risk feature such as stage Ic, II or grade 3, tumours.

In a study (24) from the Harvard Medical School, 36 stage I ovarian carcinomas were studied for p53 overexpression and it was found that the association between p53 expression and poor tumour differentiation in stage I carcinoma was statistically significant ($p = 0.03$). A study from the Mayo Clinic (25) presented results of p53 overexpression in patients with stages I and II disease. In a subset of 36 stage I patients, p53 overexpression approached statistical significance as a negative prognostic factor in univariate analysis, but in multivariate analysis it did not remain as an independent prognostic factor. The authors recommend that the p53 expression should be studied in a large cohort of early stage patients where accurate prognostic information is needed for direct therapy. In an Italian study from Milan by Bosari et al. (26), a p53 accumulation showed a trend towards a shorter duration of survival in early stage of the disease, but this did not reach statistical significance. This result is in accordance with our present results of a numeric trend towards a correlation between overexpression of p53 and decreased survival. This was also true for the subset of cases showing p53 overexpression without detectable WAF-1/p21 expression.

In a recently published study from Germany the investigators used oestrogen receptors, progesterone receptors, DNA ploidy, S-phase fraction, Ki-67, C-erbB2 onco-

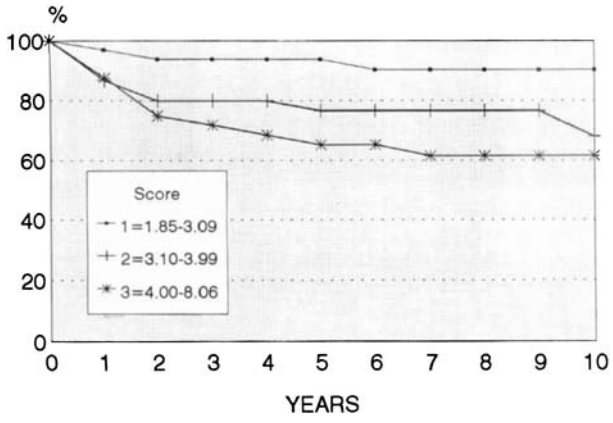

Fig. 5. Correlation between risk score, based on biological markers and survival

protein, EGF-F, cathepsin D and p170 glycoprotein as new biological parameters. Their study included tumour tissue from 77 patients with ovarian cancer, most of them in advanced stage. Their conclusion was that a combination of markers could improve the prognostic significance (27). However, in ovarian carcinoma, patients in the advanced stages usually have to undergo multimodality treatment in any case and therefore prognostic tools seldom provide an improved therapeutic approach. In contrast, our own study included only patients with early-stage disease, where it is of importance to have a non-invasive prognostic instrument in order to avoid adjuvant therapy.

The investigation of DNA, MIB, p53 and WAF-1 has shown a prognostic trend, although not always significantly, when evaluated independently. In such a situation the sum of the results from these markers could very well show a significant correlation to survival—as here. This could be explained by the fact that in the sum several factors work together to form a new factor (the score), in contrast to studying them individually.

Our results suggest that a combination of DNA, MIB-1, p53 and WAF-1 can be used as predictor to distinguish between low and high risk patients and that patients with a good prognostic score can, it is hoped, avoid adjuvant therapy after surgery.

Since tumour progression is a multistep process, most probably including a multiplicity of cellular events, our future investigations of early-stage ovarian carcinoma will concentrate on the additive prognostic value of markers such as angiogenesis, specific types of adhesion molecules and metalloproteases.

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