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DNA content evaluation for epithelial ovarian cancer identification

Analiza zawartości DNA w rozpoznaniu nabłonkowego raka jajnika

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Abstract

Objective: To assess the cellular DNA status of epithelial ovarian cancer cells for clinical stage identification and its effect on survival. **Methods:** Sixty-two patients treated by primary surgery and six courses of platinum-based chemotherapy were enrolled. The surgical stage was analyzed in correlation with DNA ploidy, S-phase fraction and DNA index. DNA analysis was performed via image cytometry. **Results:** From the 62 cases, 38 were International Federation of Gynecology and Obstetrics (Fédération Internationale de Gynécologie et d'Obstétrique, FIGO) stage I and II, 24 – stage III and IV. In the DNA histograms obtained, the DNA index ranged from 0.85 to 3.02. Sixteen were classified as diploid and 46 as aneuploid (18 multiploid). S-phase fraction ranged from 9.8 to 51%. The aneuploid cells with DNA content above 5C ranged from 0.0 to 77.2%. Patients diagnosed with FIGO III and IV (vs. I and II) were 3.3 times more likely to die. Only in FIGO stage I and II the survival differed significantly for the different groups of ploidy. The risk of death for the multiploid (vs. diploid) group is 6.4 times and for aneuploid (vs. diploid) 2.3 times. Overall survival was better in the group with low DNA index. The low percentage compared with a high percentage of 5C cells ploidy groups showed association with mortality. The death hazard for the S-phase >33 median group is 4.9 times the hazard in relation to the S-phase <33. **Conclusions:** DNA ploidy, DNA index, S-phase, and 5C cells are important prognosticators for epithelial ovarian cancer mainly in early stages.

Keywords: DNA content, epithelial ovarian cancer, prognosis

Streszczenie

Cel: Ocena statusu DNA komórek nabłonkowego raka jajnika w różnych stopniach zaawansowania klinicznego i jego wpływ na przeżycie. **Metoda:** Do badania zakwalifikowano 62 pacjentki leczone operacyjnie i za pomocą chemioterapii opartej na platynie (6 kursów). Stopień zaawansowania klinicznego nowotworu analizowano w odniesieniu do ploidalności DNA, frakcji fazy S oraz indeksu DNA. Analizę DNA przeprowadzono z zastosowaniem cytometrii obrazowej. **Wyniki:** Spośród 62 pacjentek 38 zakwalifikowano jako stopień zaawansowania I i II, natomiast 24 – jako stopień III i IV wg FIGO (International Federation of Gynecology and Obstetrics). W otrzymanych histogramach DNA indeks DNA wynosił 0,85–3,02. Szesnaście przypadków raka zaklasyfikowano jako diploidalne, natomiast 46 – jako aneuploidalne (18 multiploidalnych). Frakcja fazy S mieściła się w przedziale 9,8–51%. Odsetek komórek aneuploidalnych z zawartością DNA powyżej poziomu 5C wynosił 0,0–77,2%. Ryzyko śmierci było 3,3-krotnie większe w przypadku pacjentek z chorobą w stopniu zaawansowania FIGO III i IV (w porównaniu z I i II). Jedynie w przypadku stopni zaawansowania FIGO I i II odnotowano istotne różnice w przeżywalności między poszczególnymi grupami ploidalności. Ryzyko śmierci było 6,4-krotnie większe w przypadku multiploidalności i 2,3-krotnie większe w przypadku aneuploidalności (w porównaniu z diploidalnością). Dłuższe przeżycie ogólne odnotowano w grupie o niskim indeksie DNA. Wykazano związek między niskim odsetkiem komórek 5C, w porównaniu z wysokim odsetkiem tych komórek w grupach poliploidalnych, a śmiertelnością. Ryzyko śmierci było 4,9-krotnie większe w grupie z medianą liczby komórek w fazie S >33 w porównaniu z medianą liczby komórek w fazie S <33. **Wnioski:** Ploidalność DNA, indeks DNA, faza S oraz obecność komórek 5C to ważne czynniki prognostyczne u pacjentek z nabłonkowym rakiem jajnika, głównie we wczesnym stadium.

Słowa kluczowe: zawartość DNA, nabłonkowy rak jajnika, rokowanie

INTRODUCTION

Epithelial ovarian cancer (EOC) is the fifth most common female malignancy worldwide; more than 70% of patients are diagnosed with advanced disease, and 5-year survival rates are less than 30% with approximately 141,000 new cases and 106,000 deaths annually^(1,2).

The main prognostic factors considered in the evaluation of ovarian cancer are International Federation of Gynecology and Obstetrics (Fédération Internationale de Gynécologie et d'Obstétrique, FIGO) stage, the presence of a residual tumor, histological grade, histological type, and age⁽³⁻⁶⁾. Most of them have significant shortcomings due to their subjectivity and lack of reproducibility, and low prognostic power. Therefore, the search for an additional prognostic factor that can be more objectively measured and with better reproducibility has been intensified. One of the more promising candidates in this regard is DNA content measurement⁽⁷⁻²⁶⁾. While there have been reports on a correlation between ploidy pattern and clinical pathological findings and therapeutic results, some other reports have shown no correlation between these factors⁽¹²⁾. Thus, the results are variable and no definite conclusions may be drawn.

DNA aneuploidy, which indicates a state with an abnormal DNA and chromosome content, has been identified or mentioned in various human cancers. This parameter is considered to be an important biological and prognostic variable in ovarian cancer^(17,18,22,23).

Numerous studies showed a close association between tumor ploidy and outcome (prognosis). Among patients with advanced ovarian cancer, those with diploid tumors have a significantly better survival than those with aneuploid tumors^(8,11,24).

A multivariate analysis has shown that ploidy is a powerful and independent prognosis variable. These findings have been confirmed in most studies and ploidy/DNA index (DI) is a prognostic value in patients with both early- and advanced-stage ovarian tumors as well as in those with borderline ovarian tumors^(25,26).

In the case of ovarian carcinoma, some authors have reported an objective assessment of grading by cytophotometry, achieving a good correlation between the histological grade of ovarian tumors and DNA content^(7,10). Borderline and early-stage ovarian tumors had a significantly lower number of aneuploid cells, whereas advanced-stage invarient tumors had a significantly high number of aneuploid cells.

Image cytometry studies report that it is possible to determine the ploidy level in a given tumor using the parameters obtained from DNA histogram, assessed based on Feulgen-stained nuclei. The percentage of aneuploid cells with DNA content above 5C (5CER) is another ploidy parameter and is important in assessing ovarian cancer aggressiveness, being a reliable biomarker in the risk assessment for this type of tumor⁽²³⁻²⁶⁾.

This aim of the study was to evaluate the value of DNA content (DNA ploidy/DI/S-phase, 5CER), using image

cytometry, for predicting long-term survival of patients with epithelial ovarian cancer.

MATERIAL AND METHODS

The study comprised 62 patients with ovarian cancer diagnosis who were treated from January 1982 to December 1997 in the Hospital Geral de Santo Antonio, Porto, Portugal.

All patients were treated by multidisciplinary medical surgical teams and following international protocols (defined by the corresponding FIGO stage), and were subjected to chemotherapy based on platinum when indicated.

All patients had invasive tumors. All histological sections were reviewed by a reference pathologist and histological classifications were performed using the criteria defined by the World Health Organization (WHO). The tumors were graded according to the WHO histological grading system as grade 1, 2 or 3. Clinical information was available for all patients (date of initial diagnosis, surgical stage, tumor grade, initial tumor volume, residual tumor volume, histological differentiation, age, treatment, follow-up) and the date of death confirmed. The study ended in June 2006.

Quantification of DNA content

Ovary sections of 6 μ m were cut from paraffin-embedded blocks, deparaffinized and rehydrated. The slides were then Feulgen-stained with CAS DNA Staining Kit (Cell Analysis Systems, Elmhurst, IL), according to the manufacturer's instructions. Briefly, the sections were hydrolyzed in 5N HCL solution for 60 minutes and then transferred to the CAS DNA stain solution for 1 hour. After staining, the slides were placed in three consecutive CAS rinse solutions for 30 seconds, 5 minutes and 10 minutes, respectively. The slides were then washed in deionized water and placed in acid alcohol solution for 5 minutes. Finally, the slides were dehydrated and mounted. The Feulgen reaction produced a blue staining of nuclear DNA reflecting stoichiometric binding of the stain to the DNA. CAS staining of rat hepatocytes was used as a control slide. These cells, having a known quantity of DNA, were used as the external control, allowing for instrument calibration prior to DNA image analysis. The nuclear DNA content of cells was measured with the CAS 200 image analysis system (Cell Analysis Systems, Elmhurst, IL). The quantitative DNA analysis system is well suited for DNA content evaluation in human cells. However, if the diploid value of the specimens studied is different from human diploid cells, an "internal control" must be used, such as lymphocytes. For this reason in each tumor in addition to a minimum of 130 non-overlapping and well preserved ovarian nuclei, 20 to 30 normal lymphocytes were measured. The resultant DNA histograms were analyzed by previously described methods⁽¹²⁾. For each tumor, G0/G1 peak was visually identified, mean, standard deviation (SD) and coefficient of variation (CV)

values were calculated. The DI describes the relative DNA content of the study population and was defined as the ratio of the mean DNA content of the ovarian G0/G1 peak divided by the mean DNA content of the resting diploid lymphocyte G0/G1 peak. The 5CER was also evaluated and defined as the percentage of cells with DNA values above 5n. Tumors were considered aneuploid only if a separate G0/G1 peak was distinguishable on the histogram and its DI was different from the reference lymphocyte population by more than 2 SD, mean standard deviation. A DNA diploid tumor showed a single distinct G0/G1 peak with a DI within 2 SD from the control lymphocytes and usually with less than 1% of 5CER.

Statistical analysis

The probability for the risk of death after diagnosis was calculated according to the Kaplan–Meier method. The influence of concomitant covariates on the cumulative probability rates was analysed using the proportional hazard model described by Cox.

RESULTS

All tumors were analyzed by image cytometry for ploidy, the percentage of cells in S-phase, and 5CER (5C). Evaluable image cytometry DNA histograms were obtained from all 62 patients with ovarian carcinoma. The DI ranged from 0.85 to 3.02. Sixteen (25.8%) ovarian carcinomas were classified as diploid and 46 (74.2%) as aneuploid including 18 multiploid tumors. The S-phase fraction (SPF) ranged from 9.8 to 51% with a median value of 33%. This value was not observed in 20 patients.

The percentage of 5C ranged from 0.0 to 77.2% with a median value of 3.15%.

Survival by FIGO

FIGO staging was performed in 62 patients. For the purpose of survival analysis, tumors were classified as early stage (FIGO I + FIGO II) or advanced stage (FIGO III and FIGO IV).

Patients diagnosed with FIGO III and IV were 3.3 times more likely to die than patients with FIGO I and II (95% confidence interval, CI: 1.667–6.564).

Survival for types of ploidy by stage of FIGO

It was shown (Fig. 1) that only in FIGO I and II the survival differed significantly for the different groups of ploidy:

I + II: *df*: 2 Log-rank: 7.450 *p*-value = **0.024**

Breslow: 8.484 *p*-value = **0.014**

III + IV: *df*: 2 Log-rank: 0.610 *p*-value = 0.737

Breslow: 0.528 *p*-value = 0.768

The hazard for the multiploid group is 6.4 times the risk of death in relation to the diploid group (95% CI: 1.283–32.171) with statistical significance (*p* = 0.024).

The hazard for the aneuploid is 2.3 times the risk of death for the diploid, without significance (95% CI: 0.474–11.015).

Survival for DNA median value

Considering the test in each stratum (Fig. 2) it can be seen that for FIGO I and II the survival does not differ significantly between the groups of DNA, but the curves are well separated:

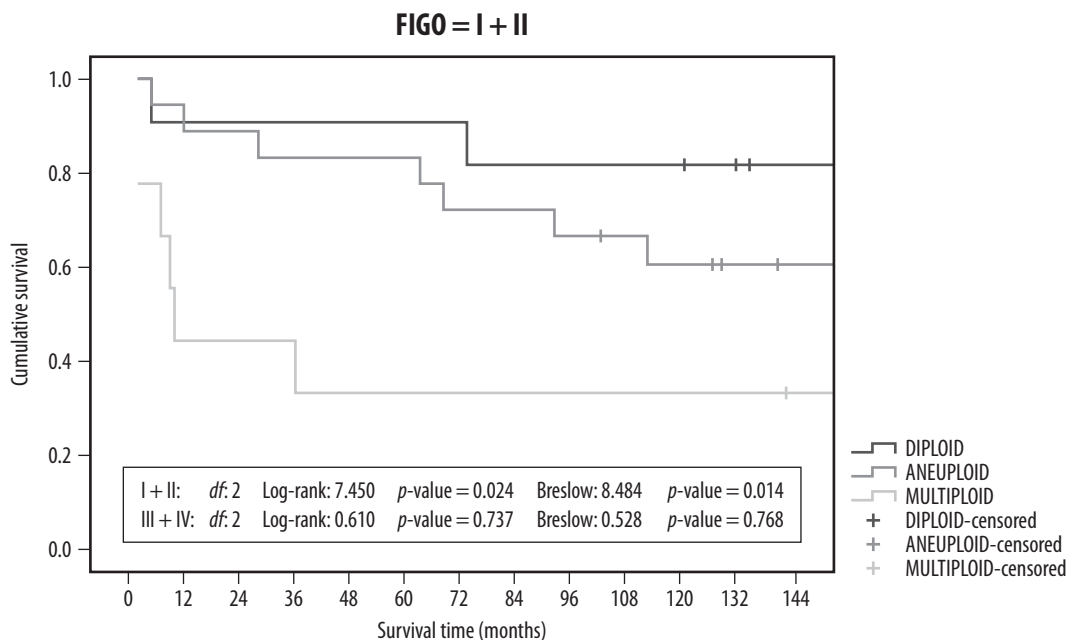


Fig. 1. Epithelial ovarian cancer: early and advanced stages and cumulative survival by ploidy groups

I + II: Log-rank: $\chi^2 = 1.477$ $df = 1$ $p\text{-value} = 0.22$
 Breslow: $\chi^2 = 1.895$ $p\text{-value} = 0.17$
 III + IV: Log-rank: $\chi^2 = 0.455$ $df = 1$ $p\text{-value} = 0.50$
 Breslow: $\chi^2 = 0.182$ $df = 1$ $p\text{-value} = 0.67$

Overall, the survival is higher in the group with low DNA index (Tab. 1) compared with the group with high DNA index, however with no statistical significance.

The analysis in each stratum shows that in FIGO I and II the survival does not differ significantly between the groups of the 5C percentage (Fig. 3), but the curves are well separated:

I + II: Log-rank: $\chi^2 = 2.414$ $df = 1$ $p\text{-value} = 0.12$
 Breslow: $\chi^2 = 3.58$ $df = 1$ $p\text{-value} = 0.06$
 III + IV: Log-rank: $\chi^2 = 0.331$ $df = 1$ $p\text{-value} = 0.56$
 Breslow: $\chi^2 = 0.252$ $df = 1$ $p\text{-value} = 0.62$

Results for the low percentage of 5C cells ploidy groups (≤ 3.15) compared with high percentage of 5C cells (> 3.15) (Tab. 2 and Fig. 4) showed association with mortality.

After approximately five years, the survival was higher in patients with S-phase ≤ 33 (Fig. 4).

I + II: Log-rank: $\chi^2 = 4.795$ $df = 1$ $p\text{-value} = 0.03$
 Breslow: $\chi^2 = 4.837$ $df = 1$ $p\text{-value} = 0.03$
 III + IV: Log-rank: $\chi^2 = 0.013$ $df = 1$ $p\text{-value} = 0.91$
 Breslow: $\chi^2 = 0.04$ $df = 1$ $p\text{-value} = 0.84$

The hazard for the S-phase > 33 (median) group is 4.9 times the risk in relation to the S-phase < 33 (95% CI: 1.009–23.572) with statistical significance ($p = 0.049$).

The clinical stage was the strongest covariate, with FIGO III and IV patients being, 3 times more likely to die than patients with FIGO I and II (95% CI: 1.571–5.610).

DISCUSSION

Ovarian cancer is the leading cause of death from gynecologic malignancies in the Western world. The majority of patients are diagnosed with advanced stage disease primarily due to peritoneal seeding from the primary tumor, which leads to peritoneal carcinomatosis without specific signs or symptoms. In addition, screening strategies have not yet been proven effective in increasing the number of patients diagnosed at earlier stages^(2,27). Approximately one-third of the presently studied cases were localized (early stage disease); the prognosis for these women was much better than for patients with FIGO stage III or IV (two-third of the cases). Combined therapy with surgery followed by chemotherapy has been the cornerstone of therapeutic management in the past two decades.

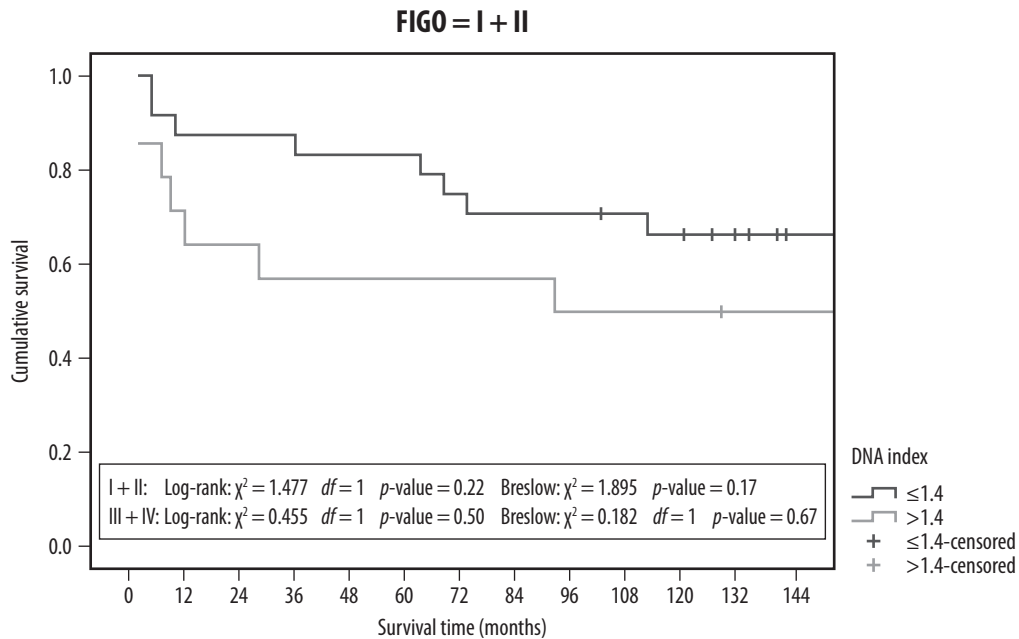


Fig. 2. Epithelial ovarian cancer: cumulative survival by DNA index groups

FIGO		N	% censored	Median	95% median confidence interval	Upper quartile
I + II	Perc5C ≤ 3.15	22	68.2	-	-	92
	Perc5C > 3.15	16	50.0	28	-	7
III + IV	Perc5C ≤ 3.15	9	22.2	19	0.000–42.374	8
	Perc5C > 3.15	15	13.3	14	7.688–20.312	8

Per5C – percentage of 5C.
 Log rank: $\chi^2 = 2.102$, $df = 1$, $p\text{-value} = 0.15$; Breslow: $\chi^2 = 3.557$, $df = 1$, $p\text{-value} = 0.06$.

6 Tab. 1. FIGO I and II cumulative survival of 5C cells groups

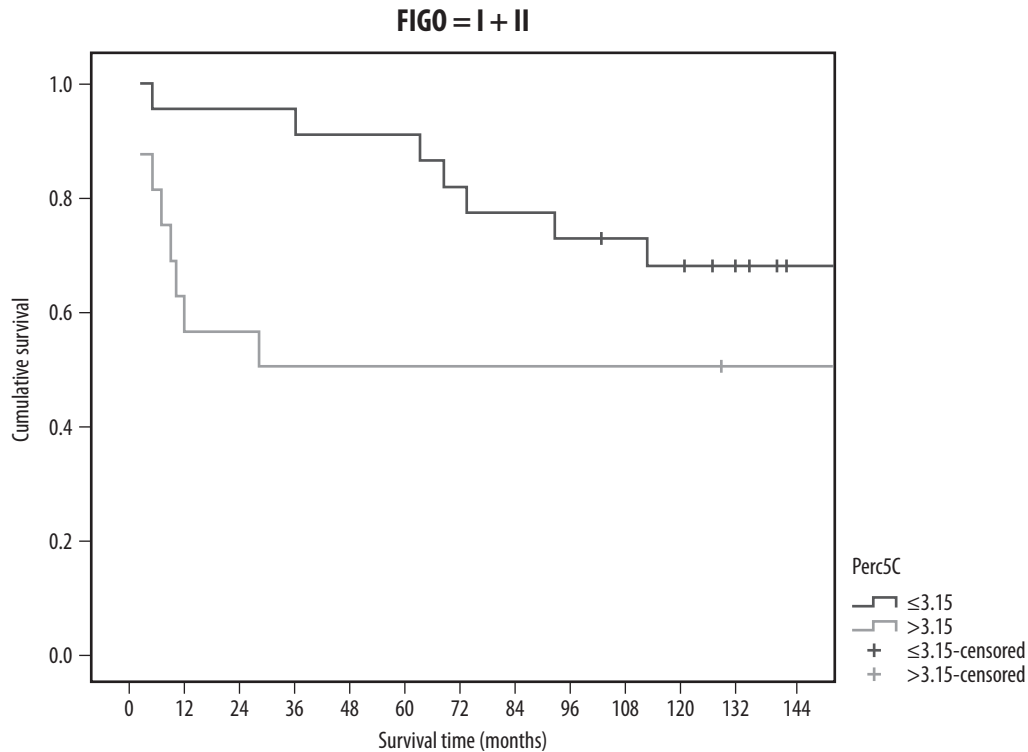


Fig. 3. Epithelial ovarian cancer cumulative survival: low vs. high percentage of 5C cells groups

Substantial progress has been made and although an increasing number of patients live longer with their disease, the majority of patients with advanced ovarian cancer are not cured. The prognosis in ovarian cancer is discouraging compared to other malignancies of the female genital tract. Despite aggressive surgery and intensified chemotherapy, the outcome of patients with stage III and IV is poor. Relevant prognostic factors are necessary to estimate the course of the disease and to define biologically similar subgroups for the analysis of therapeutic efficacy^(10,28). The prognosis of patients with ovarian cancer was suggested to depend on individual tumor characteristics rather than on therapy^(10,13). Many studies have been devoted to finding “prognostic factors,” and numerous features have been described that can help predict the prognosis of early and advanced ovarian cancer with varying degrees of accuracy. One of the most promising candidates in this regard is DNA content measurement. In the early 1970s, DNA ploidy in ovarian cancer was suggested to be of prognostic impact. It has been confirmed in most studies that DNA content (DNA ploidy/S-phase/DI/5C) is of prognostic value in patients with both early and advanced stage ovarian tumors as well as in those with borderline ovarian tumors^(15,17,18,25,29–31), results that we could associate with recent EGFL7 expression studies⁽³²⁾.

In this study, unlike in the great majority of other studies, the percentage of early stage (I–II) cancers was higher than the advanced stage cancers. This is due to the fact that many of these cases were referred to hospital.

Most of the patients were diagnosed in routine gynecologic exams or by other specialists after imaging exams for different clinical indications. In fact, nowadays, an increased number ovarian cancers are diagnosed at early stages, also as a result of improved primary care provided by general practitioners. Nevertheless, we still diagnose a great number of advanced stage cancers, although this number is decreasing. In our study, 38 cases were FIGO stages I and II and 24 cases were stage III and IV. Concerning the survival rate, groups I/II and III/IV were formed. In the first group, the survival rate was 3.3 times higher when compared with the second one. The stage is considered the most important prognostic factor in epithelial ovarian cancer. There are subgroups of patients with significant differences in survival rates: patients with surgically documented early disease (localized) versus patients with advanced disseminated disease. The 5-year survival rate is about 70–90% for the first groups and 10–18% for the second one, confirming the results of different studies^(27,32).

The survival rate in relation to ploidy and stages showed that significant differences were present only in FIGO stages I and II, for the different ploidy groups. The group of aneuploid tumors was most frequent. The frequency of aneuploidy increases, among other things, with age, advanced disease, histologic type⁽³⁾. As in other studies, the non-diploid tumors were more frequent in advanced stage tumors. The survival rate for diploid tumors was 4–6 times greater when compared with multiploid tumors. So, in the same stage, the DNA ploidy showed

FIGO		N	% censored	Median	95% median confidence interval	Upper quartile
I + II	S-phase ≤ 33	14	85.7	-	-	-
	S-phase > 33	13	46.2	112	-	63
III + IV	S-phase ≤ 33	11	9.1	15	2.053–27.947	8
	S-phase > 33	4	25.0	11	0.000–25.700	5

Log rank: $\chi^2 = 2.095$, $df=1$, p -value = 0.15; Breslow: $\chi^2 = 4.38$, $df=1$, p -value = 0.036.

Tab. 2. FIGO I + II and FIGO III + IV cumulative survival of S-phase groups

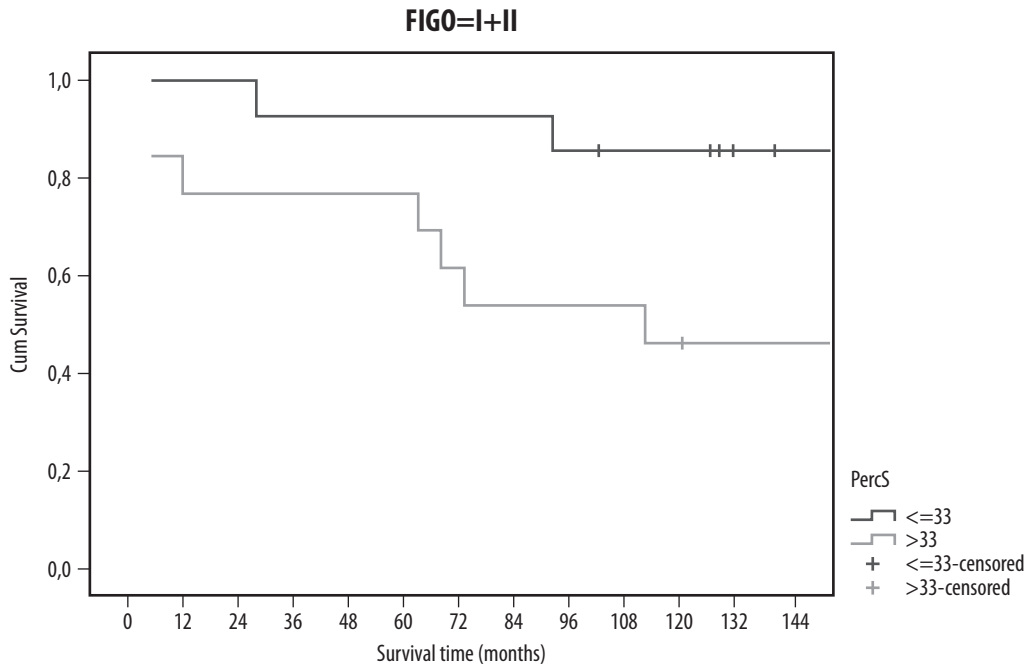


Fig. 4. Epithelial ovarian cancer cumulative survival: S-phase >33 (median) vs. S-phase <33 groups

to be an inadequate prognostic factor, with lower survival rates for the non-diploid tumors than the diploid tumors. Tropé et al. showed that in FIGO stage I DNA ploidy was a significant independent prognostic factor for disease-free survival with $p < 0.0001^{(22)}$. It was reported that a DNA index of more than 1.3 was the most important prognostic factor in multivariate analysis⁽²⁹⁾, in contrast to the report of Friedlander et al., who found that in the group of DNA-periploid tumors only the exact DNA-diploid tumors are relevant for a better prognosis^(8,9). In this investigation, tumors with DNA index less than 1.4 had better survival rate compared with tumors with a DNA index higher than 1.4, although no statistical difference was observed in this study. Tumors with a higher DNA index have a more aggressive biological nature^(33–37). There is a correlation between DNA ploidy/DI and other factors, such as histological grade, with higher aggressiveness in high grade tumors. Aneuploid tumors were more often poorly differentiated than euploid tumors. Multiploid tumors differ in survival rate, with lower survival versus diploid tumors. Patients with aneuploid

tumors with a high percentage of 5C cells (multiploid) had significantly poorer 2 and 5-year corrected survivals than patients with diploid tumors. In this study, the tumors with 5C ploidy lower than 3.15 showed association with better survival rates when compared to tumors with an elevated percentage of 5C cells, with advanced stage cancers having a higher number of 5C cells. The cell kinetic data are also important indicators of tumor aggressiveness and treatment response. Tumor growth is determined by a balance between tumor cell proliferation and cell loss, and proliferation is, in turn, dependent on the fraction of proliferating cells and cell cycling time. Determination of ovarian tumor proliferating activity will be of prognostic value. Several methods exist for this purpose, including Ki-67 expression and cytometric SPF. DNA ploidy and SPF showed a significant correlation with survival in malignant tumor, while SPF > 10% showed significant correlation with decreased survival in stage I and II cancer^(29,30,38). There are some data to suggest that the SPF may be of prognostic significance in ovarian cancer, but there are also a number of studies

that contradict these results⁽²⁵⁾. Coley et al. published that tumors with a high SPF showed shorter median survival compared to those with a low SPF⁽¹³⁾. The aneuploid tumor also showed the highest SPFs, which was indicative of a high proliferation rate⁽¹³⁾. In Norwegian Radium Hospital series, the diploid tumors had a significantly lower SPF than the aneuploid tumors. One of the reasons why the results from previous works have been controversial is probably the tumor heterogeneity^(30,39). In our study patients with S-phase ≤ 33 had greater survival rate when compared with patients with S-phase >33 , with a risk of survival less than 4.9 showing statistical significance in the present study.

CONCLUSION

In this study we can conclude that all the variables that differed in mortality occurred in patients with early stage cancers (I and II), although these are increasingly detected with higher sensibility and specificity of screening using CA-125 plus HE4 and biomarkers^(2,27,28). In these stages, the multiploid tumors with elevated S-phase showed a predictive value for higher risk of mortality.

The DNA content is an important prognostic factor for epithelial ovarian cancer. Other important factors like residual disease, age, histology type and grade were not evaluated as well as other immunohistochemical factors including ARID1A, CA-125 and its new cut-off of 18 U/mL^(32,40). The study of these other factors would contribute to a better understanding of this cancer.

Conflict of interest

No potential conflict of interest relevant to this article was reported.

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To all contributors for this paper, we must register our acknowledgment.

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