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Ovarian cancer stage I: DNA ploidy and relations with clinicopathological features

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Objectives. To assess the cellular DNA ploidy status of stage I ovarian cancers with regard to clinicopathological findings.

Materials and methods. Forty paraffin-embedded ovarian archival tumours were analyzed by flow cytometry. The DNA ploidy of the tumours was studied and related to other known prognostic factors (age, histopathologic subtype, tumour grade, survival and recurrence status). Statistical analysis was perfomed using the Statistica for Windows statistical package (Version 5.5A). Associations between DNA ploidy and clinicopathological variables were tested by the standard chi-square test. Differences between the mean values were analyzed using the Student's t test. P values below 0.05 were considered significant.

Results. Aneuploidy (DNA index >1.1) was established in 20 (53%) tumours. The mean DNA index value in the group of aneuploid tumours was 1.7. The patients' age was significantly different (p = 0.001) in the group with DNA diploid (51.6 ± 6.9 years) and aneuploid (62.5 ± 7.9 years) tumours. Nine from 14 serous tumours (64%), four from five clear cell (80%) carcinomas, three from four mucinous tumours (75%), and 50% of endometrioid tumours showed an aneuploid DNA profile. Seven (100%) ovarian granulosa cell tumours were diploid. DNA ploidy was significantly associated with tumour grade and histopathological subtype. Tumours with DNA aneuploid populations tended to have a worse prognosis than diploid tumours, although the diferrence did not reach statistical significance.

Conclusions. DNA ploidy may complement conventional histopathological diagnosis by providing an objective parameter that correlates with the biological behavior.

Key words: flow cytometry, ovarian cancer, DNA ploidy

INTRODUCTION

The main prognostic factors in early ovarian cancer are FIGO stage, histologic grade, histologic type, and age (1–3). Most of them have significant shortcomings due to their subjectivity and lack of reproducibility (4), and low prognostic power. Therefore, the search for additional prognostic factors that can be more objectively measured with better reproducibility has intensified. One of the more promising candidates in this regard is DNA content measurement (5, 6). While there have thus been reports on a correlation between ploidy pattern and clinicopathological findings or therapeutic results (7–10), some reports have shown no correlation among these factors (11, 12). Thus, the results have been variable, and no definite conclusions have yet been drawn. On the other hand, because full standardization of flow cytometry DNA analysis is still lacking, different prognostic results related to DNA ploidy may be found in various countries at different levels of the flow cytometric methodology used: how many nuclei have to be measured with flow cytometry for reliable DNA histogram interpretation (13), a difference in the processing of tissue specimens, *i.e.* frozen, fresh or paraffin-embedded (14), inadequate sampling, *e.g.*, by inclusion of major benign, necrotic or heavily inflamed poor areas (4).

As a first step towards incorporation of a biological marker such as ploidy into routine clinical practice, we performed flow cytometric DNA analyses on archival material from primary ovarian tumours and compared the DNA status (ploidy and DNA index) with other clinicopathological features.

MATERIALS AND METHODS

The eligibility criteria for the patients included was FIGO stage I primary ovarian tumours and the availability of representative samples for flow cytometry. The exclusion criteria included previous treatment

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with chemo- or radiotherapy and previous or concomitant other malignant disease.

The study group consisted of 40 women with primary ovarian cancer stage I diagnosed at the Lithuanian Oncology Center between 1999 and 2000.

The most frequent type of cancer was serous (n = 14 or 37%), followed by endometrioid (n = 8 or 21%), granulosa cell type (n = 7 or 18%), clear cell carcinoma type (n = 5 or 13%), mucinous type (n = 4 or 11%). The degree of differenciation was the following: grade 1 (G1), n = 15 (39%); grade 2 (G2), n = 9 (24%); grade 3 (G3) n = 8 (21%), and not graded n = 6 (16%).

DNA FLOW CYTOMETRY STUDY

DNA flow cytometry was performed from formalin-fixed paraffin-embedded tissue blocks. Nuclei were extracted from paraffin-embedded tissue using a modification of the Hedley technique (15). Three to five 50 μm sections were cut from suitable tumour blocks, deparaffinized in xylene, and rehydrated in 100% isopropanol and a series of solutions with decreasing concentrations of ethanol (95%, 70% and 50%) and finally in distilled water at room temperature. The rehydrated slices were suspended in 0.5% pepsin (Sigma Chemical Co.) in 0.9% NaCl, pH 1.5, and incubated at 37 °C with intermittent agitation to produce a nuclear suspension. Isolation of nuclei was revealed by microscopic observation after 30 to 60 min of incubation. After filtration through a 50 μm nylon mesh, the nuclei were washed twice in PBS and then centrifuged. The nuclear pellet was resuspended in a 0.1% NP40-trisodium citrate solution (16) and treated with RNase solution (40 μg/ml). Finally the nuclear DNA was stained with propidium iodide (50 μg/ml). The samples were analyzed on a FACSort cytometer (Becton Dickinson, Heidelberg, Germany). Red fluorescence (propidium iodide = DNA content) was collected in an FL2 detector. Data from 30,000 nuclei per sample were acquired as DNA content histogram using LYSYS II software (Becton Dickinson, Heidelberg, Germany). Doublets were excluded by using the doublet discrimination mode.

DNA HISTOGRAM INTERPRETATION

WinMDI version 2.8 software was used to analyse the acquired data. Tumours with only one G0/G1 peak were designed as diploid, and those with histograms suggesting more than G0/G1 population were categorized as aneuploid. The relative DNA content (ploidy level) was expressed as a DNA index (DI). The DI was calculated as the ratio of aneuploid to diploid G0/G1 peak channel on a histogram. Tumours with a DI = 1 ± 0.1 were defined as DNA-diploid, and tumours with higher DI values were considered DNA-aneuploid. Diploid cell populations in a sample were used as the internal standard. Cases were not considered suitable for analysis if the ovarian sample did not have enough cells, if the histogram was not interpretable, or if the coefficient of variation (CV) > 10%.

For quantitative DNA analysis, two samples demonstrated a CV > 10%. Therefore, they were eliminated from the analysis.

STATISTICAL ANALYSIS

Statistical analysis was performed using the Statistica for Windows statistical package on a personal computer. Possible associations among the variables (ploidy, tumour grade, histological subtype, survival and recurrence status) were determined using the χ^2 test. Differences among the mean values were analyzed using the Student's t test. Only $p < 0.05$ was considered statistically significant.

RESULTS

In the 2-year period, 40 patients with primary ovarian cancer were included in our analysis. The average age of patients was 57.1 ± 10.9. The patients' age was significantly different (p = 0.001) in the groups with DNA diploid (51.6 ± 6.9 years) and aneuploid tumours (62.5 ± 7.9 years).

Of the 40 paraffin-embedded ovarian tumours analyzed in the present study, DNA ploidy was obtained from 38 samples. The ploidy and survival status with respect to clinicopathologic features is shown in Table.

DNA ploidy (diploid *versus* aneuploid) was significantly associated with the histopathological subtype and tumour grade. Aneuploidy (DNA index >1.1) was established in 20 (53%) tumours. Nine from 14 serous tumours (64%), four from five clear cell (80%) carcinomas, three from four mucinous tumours (75%), and 50% endometrioid tumours showed an aneuploid DNA profile. Seven (100%) ovarian granulosa cell tumours were diploid. Seventy-five percent of poorly differenciated (G3) carcinomas were aneuploid *versus* 53% of those well-differenciated (G1).

The mean follow-up in surviving patients was 42 months (range, 4–64 months). Recurrence was observed in one of the 18 patients with diploid tumours and in five of the 20 patients with aneuploid tumours, four from whom were serous subtype. Neither of these differences was statistically significant. The 5-year survival rates of patients with diploid and aneuploid tumours were 94% and 77%, respectively.

The DNA index values counted for 38 paraffin-embedded ovarian specimens ranged from 1.0 to 2.5. (Figure). The mean DNA index value in the group of aneuploid tumours was 1.7. The average DI of serous, endometrioid, clear cell and mucinous aneup-

Table. **DNA ploidy *versus* clinicopathologic features**

Clinicopathologic features	DNA ploidy distribution		p value
	Diploid	Aneuploid	
Histopathologic type			0.028 (λ^2)
serous	5	9	
endometrioid	4	4	
mucinous	1	3	
clear cell	1	4	
granulosa cell	7	–	
Tumour grade			0.029 (λ^2)
G1	8	7	
G2	2	6	
G3	3	6	
not graded	6	–	
Survival status			0.188 (λ^2)
dead	1	4	
alive	17	16	
Recurrence	2	5	0.270 (λ^2)
Age (mean), years	51.6 ± 6.9	62.5 ± 7.9	0.001 (t test)

loid tumours were 1.7 ± 0.2, 1.5 ± 0.2, 2.1 ± 0.4 and 1.4 ± 0.3, respectively.

DISCUSSION

To the best of our knowledge, this is the first study in which flow cytometric DNA analysis has been carried out on ovarian tumours in Lithuanian patients. In this study, we investigated the DNA ploidy pattern in 38 primary ovarian tumours. As there are five tumour type categories, four histological grades and two variables (diploid and aneuploid), this gives a theoretical total of 40 possible combinations of these variables. The number of patients in this study is inadequate to consider all possible combinations. Because of the small number of cases used in this study, the DNA ploidy parameters nessesary for the statistical evaluation are grouped in simplified terms which are diploid and aneuploid.

Patients with aneuploid tumours were older than those with diploid tumours. This finding was of no surprise, because ovarian cancer is most common in menopausal women over 50 years of age (17). The study revealed that the frequency of aneuploid DNA tumours was 53%, which is consistent with more recent findings (18, 19). DNA ploidy (diploid *versus* aneuploid) was significantly associated with tumour grade and histopathological subtype.

The present results emphasize the greater frequency of aneuploidy in clear cell and serous carcinomas, consistent with previous studies by Skirnisdottir et al. (18). It is interesting to note that serous and clear cell tumours, which are suggested to have a more aggressive biological nature compared to the other types (21), tended to have a higher DNA index (mean 1.7 and 2.1, respectively). Schueler et al. (22) found that DI > 1.40 could be used to discriminate between high and low risk aneuploid stemlines. These observations are in contrast to several studies (23), defining endometrioid and serous ovarian cancers to have a better prognosis than mucinous and clear cell ovarian cancers. The subjectivity of typing is likely to account, at least in part, for the different results generated from studies undertaken to date.

It does not appear feasible to predict the clinical course of granulosa cell tumours by DNA flow cytometry. However, it can be concluded that most granulosa cell tumours have diploid DNA content. Miller et al. (20) reported that ploidy in granulosa cell tumours of the ovary did not predict outcome. Estimation on a larger study group is, however, needed.

In this study, no correlation was found between DNA ploidy and the survival or recurrence rate but, in view of the small numbers and limited follow-up, we cannot definitely analyze these prognostic factors. Although our results do not provide evidence to indicate clearly the merit of using tumour ploidy for discriminating between good-risk and poor-risk populations of ovarian cancer patients, they cannot be used to dismiss further evaluation of the value of such marker.

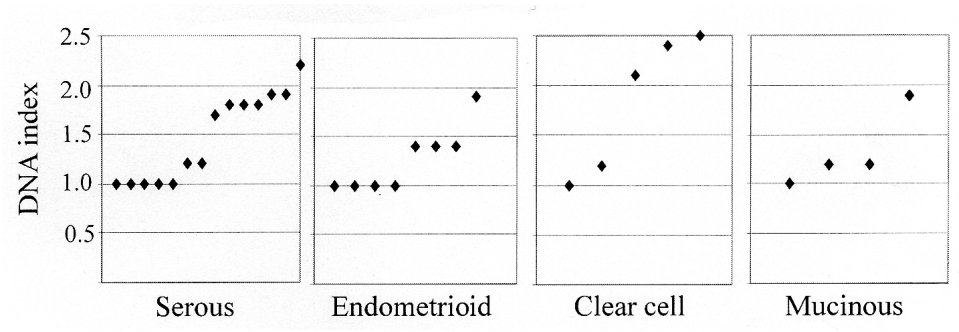

Figure. Distribution of DNA index values in different histopathological subtypes

In conclusion, DNA ploidy may complement the conventional histopathological diagnosis by providing an objecti-

ve parameter that correlates with the biological behavior. However, studies should be continued to prove results on the major number of patients and more homogeneous patients' groups with respect to tumour type and grade.

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References

1. Vergote I. Prognostic factors in stage I ovarian carcinoma. Verh K Acad Geneeskd Belg 2001; 63: 257–71.
2. Clark TG, Stewart ME, Altman DG, Gabra H, Smyth JF. A prognostic model for ovarian cancer. Br J Cancer 2001; 85: 944–52.
3. Silverberg SG. Histopathologic grading of ovarian carcinoma: a review and proposal. Int J Gynecol Pathol 2000; 19: 7–15.
4. Baak JPA, Janssen E. DNA ploidy analysis in histopathology. Histopathology 2004; 44: 603–20.
5. El-Naggar AK, Vielh P. Solid tumour DNA content analysis. Methods Mol Biol 2004; 263: 355–70.
6. Ozalp S, Yalcin OT, Gulbas Z, Tanir H.M, Minsin T. Effect of cellular DNA content on the prognosis of epithelial ovarian cancers. Gynecol Obstet Invest 2001; 52: 93–7.
7. Mecke H, Borner-Klaussner B, Grosse G, Nadjari B, Hauptmann S. Clinical behaviour of serous and mucinous bordeline tumors of the ovary with diploid DNA stemline. Follow-up studies after organ preserving and non-organ preserving therapy. Zebtralbl Gynakol 2000; 122: 274–9.
8. Nagai N, Oshita T, Fuji T, Katsube Y, Matsubayashi S, Ohama K. Are DNA ploidy and epidermal growth factor receptor prognostic factors for untreated ovarian cancer? A prospective study. Am J Clin Oncol 2001; 24: 215.
9. Panichenko IV, Bogatyrev VN, Kozachenko VP, Zhordania KI. Laser DNA flow cytometry in patients with ovarian epithelial tumours. Klin Lab Diagn 2002; 4: 48–51.
10. Kim YT, Zhao M, Kim SH, Lee CS, Kim JW. Prognostic significance of DNA quantification by flow cytometry in ovarian tumours. Int J Gynecol Obstetrics 2005; 88: 286–91.
11. Karaburun S, Karaveli S, Pestereli HE, Simsek T, Sargin CF. Proliferating cell nuclear antigen (PCNA) and Ki-67 in epithelial ovarian tumours: correlation with DNA flow analysis. Adv Clin Path 2001; 5: 3–9.
12. Coley HM, Sargent JM, Titley J, Taylor CG. Lack of prognostic significance of ploidy and S-phase measurements in advanced ovarian cancer. Anticancer Res 1999; 19: 2111–6.
13. Bergers E, van Diest PJ, Baak JP. Reliable DNA histogram interpretation. Number of nuclei requiring measurement with flow cytometry. Anal Quant Cytol Histol 1997; 19: 277–84.
14. Silvestrini R. Relevance of DNA ploidy as a prognostic instrument for solid tumours. Ann Oncol 2000; 11: 259.
15. Hedley DW, Friedlander ML, Taylor IW, Rugg CA, Musgrove EA. Method for analysis of cellular DNA content of paraffin-embedded pathological material using flow cytometry. J Histochem Cytochem 1983; 31: 1333–5.
16. Vindelov LL, Christensen IJ, Nissen NI. A detergent-trypsin method for preparation of nuclei for flow cytometric DNA analysis. Cytometry 1983; 3: 323–7.
17. Bj¸rge T, Engeland A, Hansen S, Tropé CG. Trends in the incidence of ovarian cancer and bordeline tumours in Norway, 1954–1993. Int J Cancer 1997; 71: 780–6.
18. Skirnisdottir I, Sorbe B, Karlsson M, Seidal T. Prognostic importance of DNA ploidy and p53 in early stages of epithelial ovarian carcinoma. Int J Oncol 2001; 19: 1295–302.
19. Curling M, Stenning S, Hudson CN, Watson JV. Multivariate analyses of DNA index, p62 c-myc, and clinicopathological status of patients with ovarian cancer. J Clin Pathol 1998; 51: 455–61.
20. Miller BE, Barron BA, Dockter ME, Delmore JE, Silva EG, Gersherson DM. Parameters of differenciation and proliferation in adult granulosa cell tumours of the ovary. Cancer Detect Prev 2001; 25: 48–54.
21. Clement PB, Young RH. Atlas of gynecologic surgical pathology. Philadelphia, London, New York, St. Louis, Toronto, Sydney; 2000; 507.
22. Schueler JA, Trimbos JB, Burg M et al. DNA index reflects the biological behavior of ovarian carcinoma stage I–II. Gynecol Oncol 1996; 62: 59–66.
23. Hornung R, Urs E, Serenella E, Edward W et al. Analysis of potential prognostic factors in 111 patients with ovarian cancer. Cancer Lett 2004; 206: 97–106.

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I STADIJOS KIAUÐIDÞIØ VËÞYS: DNR PLOIDIÐKUMAS IR RYÐYS SU KLINIKOPATOLOGINIAIS RODIKLIAIS

Santrauka

Darbo tikslas – iðtirti I kiauðidþiø vëþio stadija serganèiø ligoniø navikø DNR ploidiðkumo ryðá su klinikopatologiniais rodikliais.

Tyrimo medþiaga ir metodai. Iðtirta 40 kiauðidþiø navikø, álietø á parafinà, histologinë medþiaga. Bendras DNR kiekis làstelëse iðmatuotas tëkmës citometru. Iðtirta DNR ploidiðkumo sàsaja su kitais gerai þinomais prognostiniais veiksniais (ligoniø amþiumi, navikø histopatologiniu potipiu, diferenciacijos laipsniu, iðgyvenimu).

Duomenø statistinë analizë atlikta STATISTICA for Windows (Version 5.5A) programa. Skirtumai tarp DNR ploidiðkumo ir klinikopatologiniø poþymiø buvo ávertinti χ^2 metodu. Skirtumas statistiškai reikšmingas, kai $p < 0,05$.

Rezultatai. Aneuploidija (DNR indeksas > 1,1) buvo nustatyta 20(53%) navikø. Aneuploidiniø navikø DNR indekso vidurkis buvo 1,7. Pacienèiø amþius statistiðkai reikðmingai ($p = 0,001$) skyrësi tarp tiriamøjø grupës su diploidiniais (51,6 ± 6,9 metai) ir aneuploidiniais (62,5 ± 7,9 metai) navikais. Devyniuose ið 14 seroziniø (64%), keturiuose ið 5 ðviesialàsteliniø (80%) trijuose ið keturiø (75%) mucininiø ir 50% endometrioidiniø navikø nustatyta DNR aneuploidija. Septyni (100%) granulozolàsteliniai kiauðidþiø navikai buvo diploidiniai. Kiauðidþiø navikø DNR ploidiðkumas statistiðkai reikðmingai buvo susietas su navikø histologiniu potipiu ir diferenciacijos laipsniu.

Blogesnæ prognozæ turëjo kiauðidþiø navikai su DNR aneuploidija, lyginant su diploidiniais navikais, bet ðis skirtumas nebuvo statistiðkai reikðmingas.

Išvada. DNR ploidiðkumas suteikia papildomà objektyvià informacijà histopatologinei kiauðidþiø navikø diagnostikai.

Raktaþodþiai: tëkmës citometrija, kiauðidþiø vëþys, DNR ploidiðkumas