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AgNORs Count Correlates Better than Grading with the Effect of Chemotherapy in Serous Ovarian Cancer

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Objective: to evaluate the relationship between AgNORs count as well as tumor grade and the effect of chemotherapy in serous ovarian cancer. Material and methods: 39 women who underwent surgical procedure and then chemotherapy due to serous ovarian cancer were included into the study. In cancer cells the mean number of AgNORs per nucleus (mAgNOR) and the mean percentage of nuclei with five or more AgNORs (pAgNOR) were counted. Results: in 13 women (33.3%) we did not found the neoplastic disease in second-look laparotomy (group I), and in 26 patients (66.7%) the cancer was present (group II). The mean grading and the mAgNOR of ovarian cancer in the first operation in group I and in group II were similar. The mean pAgNOR in group I was higher than in group II. The values of mAgNOR and pAgNOR but not grading in women with the absence of cancer in second-look laparotomy and in women with the only single neoplastic focuses were similar one to another, and both were higher than in cases of disseminated neoplastic disease. Conclusions: the quantitative assessment of AgNORs is better prognostic factor when compared to grading for the effectiveness of adjuvant chemotherapy in serous ovarian cancer.

Introduction

Ovarian cancer is the most fatal gynecological cancer with the annual incidence of 15.3 per 100 000 in Poland in 1999. [8]. The most common histologic type is serous ovarian carcinoma [1, 3, 7]. Due to the asymptomatic nature of the early disease, most cases are not detected until the advanced stages [1, 3]. Consequently, more women die from ovarian cancer, than from cervical and endometrial cancer combined, and the prognosis for the patients is usually poor [8, 14, 19]. The cancer of the ovary represents the fourth major cause of all death because of malignant tumors in women after the cancer of the breast, the lung and the colon, with the mortality rate of 6.7 per 100 000 (Poland 1999) [8]. The 5-year survival rate does not exceed 30% [3, 14].

The standard of the treatment in ovarian cancer is surgery followed by chemotherapy [14, 18, 19]. These treatment methods are interdependent: adequate surgical staging is necessary to select patients, who will benefit from adjuvant chemotherapy [18]. Except of the clinical staging, histologic grading is found to be the second major independent prognostic factor. The residual disease, CA 125 level, the presence of ascites, histology, age, general condition, and various molecular markers have been reported as another factors predicting survival in patients suffering from the ovarian cancer as well [3, 14, 18, 19].

In the literature the assessment of proliferative activity of the malignant tumor has been found as the next potential independent prognostic factor [2, 4, 13, 17, 20]. The number of nucleolar organizer regions (NORs) per nucleus is a good marker of the proliferative activity of various cancers including ovarian cancer as well [2, 4, 5, 9, 12, 13, 17, 20, 26]. Nucleolar organizer regions (NORs) are segments of DNA that transcribe to ribosomal RNA, and are located on short arms of the acrocentric chromosomes: 13, 14, 15, 21 and 22. The number of NORs reflects the synthetic activity of cells, and is related to the cell cycle. The quantity of interphase AgNORs increases in cells in cycle from early G1-phase to the late S-phase. In cancer tissues the AgNOR value is also closely related to both the percentage of cycling cells and S-phase cells [6, 9, 11, 13, 22, 24]. After silver-staining, the NORs can be easily identified as black dots situated exclusively throughout the nucleolar area, and are called "AgNORs". The NORs' argyrophilia is due to a group of nucleolar proteins, which have a high affinity to silver [6, 21]. Two AgNOR counting methods have been evaluated: I - the mean number of AgNORs per nucleus (mAgNOR), which has been associated with tumor ploidy, and II - the mean percentage of nuclei with five or more AgNORs per nucleus (pAgNOR), which has been correlated with its proliferative activity [9]. The potential prognostic value of AgNORs assessment in serous ovarian cancers has not been defined clearly as yet.

The aim of the study was to investigate the correlation between tumor grading as well as the number of AgNORs

in cancer cells in initial surgery, and the quantitative effect of the adjuvant chemotherapy in ovarian cancer.

Material and Methods

Thirty-nine women aged from 28 to 76 years (mean age 53.3±10.3), who were treated between 1998 and 2002 in the I Department of Gynecology and Gynecological Oncology (Institute of Gynecology and Obstetrics, University Medical School, Łódź), due to serous ovarian cancer were included into the study. All patients were treated surgically, than chemotherapy was given. The initial surgery included bilateral adnexectomy, hysterectomy, omentectomy and appendectomy or cytoreduction only, if complete surgery was impossible. The results of treatment were assessed during second-look laparotomy. On the basis of its results we divided patients in two groups: with the absence (group I) and with the presence of cancer (group II), and additionally the group II: into patients with regressive (single small neoplastic focuses) and progressive, disseminated disease. The "single small neoplastic focuses" we diagnosed in cases when the presence of cancer was diagnosed from sections only by microscopic examination (n=3), the single neoplastic tumor up to 5cm was resected, and another focuses of disease were not found (n=4), the solitary focuses of cancer up to 2cm on the peritoneum were found without any other neoplastic focuses (n=2).

In the study, 4μm thick sections were cut from blocks containing tissue, previously routinely fixed in 10% buffered formalin and embedded in paraffin. One section was stained with hematoxylin and eosin for histopathologic diagnosis. The tumors were graded according to World Health Organization criteria and staging was done using the criteria of FIGO. The ovarian cancer at stage I was detected in 5 cases (12.8%), at stage II in 12 cases (30.8%), at stage III in 18 cases (46.2%) and at stage IV in 4 cases (10.2%). There were 9 cases (23.1%) with well-differentiated carcinoma (G-1), 11 cases (28.2%) with moderately differentiated carcinoma (G-2), and 19 cases (48.7%) with poorly differentiated cancer (G-3).

Another section from cancer tissue was stained according to „one-step AgNOR method" described by Howell and Ploton [10, 21]: specimens were incubated in a mixture of one volume of 2% gelatin in 1% formic acid and two volumes of 50% silver nitrate and then washed off 10x with deionized distilled water. Histologic morphometry was performed by means of an image analysis system consisting of a Pentium IBM-compatible computer equipped with an optical mouse, Indeo Fast card (frame grabber, true color, real time), produced by Indeo (Taiwan), and color TV camera Panasonic (Japan), linked to a microscope Jenaval-Carl Zeiss (Germany). This system was programmed (program MultiScan 8.08, produced by CSS-Poland) to calculate:

- the surface area of a structure whose perimeter was traced,
- the number of objects (automatic function with manual correction).

Both counting of AgNORs and morphometric assessment were performed at 400x magnification. AgNORs were seen as black or dark brown dots within the nucleus. The following parameters were estimated in 100 randomly chosen nuclei:

- nuclear area and nuclear outline (the outer limit of a nuclear membrane was traced out using a cursor of an optical mouse),
- the number of AgNORs per nuclear area (these objects were automatically counted and followed out with manual correction, as needed).

The correlation between the mean number of AgNORs per nucleus (mAgNOR), the mean percentage of nuclei with five or more AgNORs per nucleus (pAgNOR), as well as histologic grade of ovarian cancer and chemotherapeutic effect were studied by the Spearman rank correlation test and Student-t test for unpaired data. Calculations were performed using the CSS Statistica software (Statsoft Inc., Tulsa, OK, USA). A p value <0.05 was considered to be significant.

Results

In 13 women (33.3%) after the adjuvant chemotherapy we did not found the active neoplastic disease in second-look laparotomy. In this group in the initial surgery in 8 cases the complete surgery (hysterectomy, omentectomy, appendectomy and bilateral adnexectomy) was performed, and only in 4 cases the surgery was oncologically radical. In 9 patients (23.1%) only solitary focuses of cancer were found. In these women in the first surgery only in 2 cases the complete operation was performed, and in none case the operation was oncologically radical. In 17 cases (43.6%) the disseminated neoplastic disease was diagnosed. In the initial surgery in 4 these cases the complete surgery was performed, and only in 1 case the surgery was oncologically radical.

Basing on results of the second-look laparotomy good effect of treatment was stated in 22 cases (56.4%).

The mean mAgNOR in ovarian cancer cells from the first operation in group I was 4.71±1.01, and in group II it was 4.22±0.94 (ns). The mean pAgNOR in group I was 50.15±23.16, and it was higher than in group II, where it was 35.62±19.90 ($p=0.049$).

The values of mAgNOR and pAgNOR in women with the absence of cancer in second-look laparotomy and in women with the only single neoplastic focuses were similar, and both were higher than in cases of disseminated neoplastic disease (Tables 1 and 2). The ρ-Spearman rank correlation test values between the quantitative effect of treatment

TABLE 1

Correlation between mAgNOR in serous ovarian cancers after the first surgery and the quantitative effect of chemotherapy

Second-look laparotomy	n	%	mAgNOR	SD	Statistical analysis
absence of cancer	13	33.3	4.71	1.01	I - II - ns
only solitary focuses of cancer	9	23.1	4.74	0.78	I - III - p=0.005
disseminated disease	17	43.6	3.88	0.60	II - III - p=0.002

TABLE 2

Correlation between pAgNOR in serous ovarian cancers after the first surgery and the quantitative effect of chemotherapy

Second-look laparotomy	n	%	mAgNOR	SD	Statistical analysis
absence of cancer	13	33.3	50.15	23.16	I - II - ns
only solitary focuses of cancer	9	23.1	50.56	21.23	I - III - p=0.001
disseminated disease	17	43.6	27.71	14.20	II - III - p=0.002

TABLE 3

Correlation between histologic grade of serous ovarian cancers and the quantitative effect of chemotherapy

Second-look laparotomy	n	%	mAgNOR	SD	Statistical analysis
absence of cancer	13	33.3	2.54	0.66	I - II - ns
only solitary focuses of cancer	9	23.1	2.56	0.73	I - III - ns
disseminated disease	17	43.6	2.00	0.94	II - III - ns

and mAgNOR as well as pAgNOR were respectively ρ=-0.44 (p=0.005) and ρ=-0.51 (p<0.001). These values were both similar.

The mean grading of ovarian cancer in the first operation in group I was 2.54±0.66, and in group II it was 2.19±0.90 (ns). We found no correlation between tumor grading and the amount of neoplastic tissue found in the second-look laparotomy (Table 3). The ρ-Spearman rank correlation test value between grading and the quantitative effect of treatment was ρ=-0.32 (p=0.05) .

Discussion

Because of the poor prognosis in women suffering from ovarian cancer, there is the need of further studies on tumor biology [3, 14]. One of the most important and well defined factors in the assessment of the tumor aggressiveness is the histologic grading [14, 19]. In our material we noted higher grading in cases with the absence of the neoplasm in second-look laparotomy, than in cases with the presence of neoplastic disease, but the data were not statistically significant. The correlation between the grading of serous ovarian cancer and the quantitative effect of the adjuvant chemotherapy was nearly of statistical significance. The data confirmed well known dependency, that G-3 cancers better response to primary adjuvant chemotherapy than G-1 tumors, but the rate of recurrences is higher in G-3 cases. The disease-free time is shorter in G-3 cases, and the response to second line chemotherapy is worse as well [14, 19]. The dependency between well differentiated tumor (G-1) and the better prognosis for patients, as well as the poor differentiated tumor (G-3) and the worse prognosis for patients is confirmed for most of the malignant neoplasms, and for ovarian cancer as well [2, 14, 18]. According to results by Decker and Malkasian the time of survival in patients with G-1 ovarian cancer is longer than in patients with G-3 ovarian cancer, and the shortest time of survival is observed in group of women suffering from anaplastic primary ovarian cancer [7].

Besides the tumor grading, the proliferative activity is found to be a valuable indicator of tumor biology [5, 9, 13, 22]. Subsequently, several markers of cell proliferation and metabolic activity have been developed. The expression of nucleolar organizer regions (NORs) is the proliferative activity exponent [2, 4, 9, 11, 16]. A few reports on AgNOR count in ovarian cancer made on the small not homogenous histologic groups of tumors were published [5, 9, 12, 15, 24, 26], but we have not found reports describing the value of AgNORs assessment for the prediction of the response to chemotherapy. Our data show the significant correlation between the number of AgNORs (both mAgNOR and pAgNOR) in primary serous ovarian cancer and the quantitative result of second-look laparotomy. The higher number of nucleolar organizer regions resulted in the better response to primary adjuvant chemotherapy, and this correlation is statistically significant. Besides these data it is noteworthy, that the general prognosis for patients with high number of AgNORs is worse than for patients with low number of AgNORs [2, 13, 17]. The relation is similar to the histological grading of cancer. Muso at al. found in 37 patients with progressive ovarian cancer despite postoperative chemotherapy, the number of AgNORs significantly higher than in the group with successful treatment and concluded, that the number of NORs was considered to be useful prognostic factor in ovarian cancer [19]. The potential value of the method especially in cancers at stage Ia - Ib (the treatment

without adjuvant chemotherapy) and stage Ic (the adjuvant chemotherapy is necessary) was confirmed by Sujathan et al. [23]. They examinated 100 aspirated samples of benign and malignant effusions, and on the ground of the results concluded, that AgNORs assessment appears to be useful as an additional tool for distinguishing the character of peritoneal fluid, especially when the cytologic diagnosis is difficult. The data seem to confirm our conclusions about AgNORs assessment as the additional diagnostic tool and as the prognostic factor in ovarian cancer.

Conclusions

The quantitative assessment of AgNORs is the better prognostic factor of the effectiveness of additive chemotherapy in serous ovarian cancer compared to grading.

Mentioned above correlations, easy methods and low costs make the assessment of AgNORs potentially useful method of diagnostics and treatment of serous ovarian cancer.

References

1. *Bieńkiewicz A, Gottwald L, Suzin J:* Final staging of ovarian cancer classified during surgery as stage I (FIGO). Int J Gynecol Obstet 2000, Suppl, P3.16.15.
2. *Chiusa L, Galliano D, Formiconi A, Di Primio O, Pich A:* High and low risk prostate carcinoma determined by histologic grade and proliferative activity. Cancer 1997, 79(10), 1956-1963.
3. *Clarc TG, Stewart ME, Altman DG, Gabra H, Smyth JF:* A prognostic model of ovarian cancer. Br J Cancer 2001, 85(7), 944-952.
4. *Costa Ad, de Araujo NS, Pinto Dd, de Araujo VC:* PCNA/AgNOR and Ki-67/AgNOR double staining in oral squamous cell carcinoma. J Oral Pathol Med 1999, 28(10), 438-441.
5. *Criscuolo M, Martinelli AM, Migaldi M, Zunarelli E, Bergamaschi M, Falchi AM, De Gaetani C:* Prognostic significance of nucleolar organizer regions in ovarian epithelial tumors. Int J Gynecol Pathol 1993, 12(3), 259-263.
6. *Crocker J, Boldy D, Egan M:* How should we count AgNORs? Proposals for a standardized approach. J Pathol 1989, 158, 185-188.
7. *Decker DG, Malkasian GD, Taylo WF:* Prognostic importance of histologic grading in ovarian carcinoma. Natl Cancer Inst Monogr 1975, 42, 15-18.
8. *Didkowska J, Wojciechowska U, Tarkowski W, Zatoński W:* Malignant Neoplasms in Poland in 1999. M. Sklodowska-Curie Centre of Oncology. The National Register of Neoplasms. Warsaw 2002.
9. *Ghazizadeh M, Sasaki Y, Araki T, Konishi H, Aihara K:* Prognostic value of proliferative activity of ovarian carcinoma as revealed by PCNA and AgNOR analyses. Am J Clin Pathol 1997, 107(4), 451-458.
10. *Howell WM, Black DA:* Controlled silver-staining of nucleolus organizer regions with a protective colloidal developer: a 1-step method. Experimentia 1980, 36, 1014-1015.
11. *Jeleń M, Blok K, Blok R, Leśkow E, Gruszczyńska-Szkudlarek T:* Regions of nuclear organisations (AgNORs) and Ki-67 antigen expression from the cervix and cervical canal smears with respect to changes in ASCUS and AGUS (atypical cells of unspecified importance) according to the Bethesda cytological system of classification. Ginekol Pol 2002, 77(12), 1192-1198.
12. *Khattech A, Spatz A, Prade M, Duvillard P, Charpentier P, Bognel C, Michel G, Lhomme C:* Nucleolar organizer regions in ovarian tumors: discrimination between carcinoma and borderline tumor. Int J Pathol 1992, 11(1), 11-14.
13. *Kumar A, Kushwaha AK, Kumar M, Gupta S:* Argyrophilic nucleolar organizer regions: their value and correlation with clinical prognostic factors in breast carcinoma. J Surg Oncol 1997, 65(3), 201-204.
14. *Markowska J:* Ovarian Cancer. Springer PWN, Warszawa 1997, 9-10.
15. *Mauri FA, Scampini S, Aldovini D, Ferrero S, Barbareschi M, Dalla Palma P:* AgNOR distribution in serous tumours of the ovary. Pathologica 1990, 82(1081), 487-492.
16. *Miller B, Flax S, Dockter M, Photopulos G:* Nucleolar organizer regions in adenocarcinoma of the uterine cervix. Cancer 1994, 74 74(12), 3142-3145.
17. *Miller B, Morris M, Silva E:* Nucleolar organizer regions: a potential prognostic factor in adenocarcinoma of the endometrium. Gynecol Oncol 1994, 54(2), 137-141.
18. *Moss C, Kaye S:* Ovarian cancer: progress and continuing controversies in management. Eur J Cancer 2002, 38, 1701-1707.
19. *Muso H:* Long-term prognostic factors for chemotherapy of ovarian cancer. Osaka City Med J 1998, 44(2), 155-171.
20. *Nakae S, Nakamura T, Ikegawa R, Yoshioka H, Shirono J, Tabuschi Y:* Evaluation of argyrophilic nucleolar organizer region and proliferating cell nuclear antigen in colorectal cancer. J Surg Oncol 1998, 69(1), 28-35.
21. *Ploton D, Menager M, Jeannesson P, Himber G, Pigeon F, Adnet J:* Improvement in the staining and in the visualization of the argyrophilic proteins of the Nucleolar Organizer Regions at the optical level. Histochem J 1986, 18, 5-14.
22. *Sirri V, Roussel P, Hernandez-Verdun D:* The AgNOR proteins: qualitative and quantitative changes during the cell cycle. Micron 2000, 31(2), 121-126.
23. *Sujathan K, Kannan S, Raveendran-Pillai K, Chandralekha B, Sreedevi-Amma N, Krishnan-Nair M:* Significance of AgNOR count in differentiating malignant cells from reactive mesothelial cells in serous effusions. Acta Cytologica 1996, 40(4), 724-728.
24. *Terlikowski S, Lenczewski A, Famulski W, Sulkowski S, Kulikowski M:* Expression of nucleolar organizer regions (NORs) in ovarian epithelial tumors. Folia Histochem Cytobiol 2001, 39(2), 161-162.
25. *Wierzchniewska A, Wągrowska-Danilewicz M, Danilewicz M:* Value of AgNOR counts and morphometric analysis of nuclear parameters in premalignant and malignant lesions of the uterine cervix. Pol J Pathol 1998, 49(4), 297-301.
26. *Zergeroglu S, Aksakal O, Demirturk F, Gokmen O:* Prognostic importance of the nucleolar organizer region score in ovarian epithelial tumors. Gynecol Obstet Invest 2001, 51(1), 60-63.

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