Assessment of the argyrophilic nucleolar organizer region area/nucleus ratio in ovarian serous epithelial adenomas, borderline tumors and cancers

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Abstract

Introduction: There is a need to assess the value of the novel potentially useful biomarkers in ovarian tumors. The aim of study was to assess the value of sAgNOR analysis in ovarian serous epithelial tumors.

Material and methods: The analysis was performed in ovaries from 113 patients treated operatively due to serous ovarian tumors (30 adenomas, 14 borderline tumors and 69 cancers). After silver staining of paraffin specimens from surgery, sAgNOR in tumor cells was analyzed. Additionally, the value of the argyrophilic nucleolar organizer region area/nucleus ratio (sAgNOR) in the prediction of disease-free survival (DFS) and overall survival (OS) in 52 patients with serous ovarian cancer with complete follow-ups in November 2009 was evaluated. Age, grading, radicality of surgery and FIGO staging were analyzed as additional factors.

Results: sAgNOR in adenomas, borderline tumors and cancers was in the following ranges: (0.73 ± 0.23) × 10^6, (0.81 ± 0.18) × 10^6 and (0.96 ± 0.33) × 10^6 [AgNOR/cm^2] respectively. In cancers from G1 to G3 sAgNOR was (1.02 ± 0.32) × 10^6 (G1), (0.98 ± 0.37) × 10^6 (G2) and (0.82 ± 0.24) × 10^6 (G3) [AgNOR/cm^2] respectively. In univariate analysis, but not in multivariate analysis, staging negatively correlated with better DFS and OS. sAgNOR, age of patients, grading and radicality of surgery were not associated with DFS or OS in either univariate or multivariate analysis.

Conclusions: sAgNOR analysis is not sufficient to precisely characterize cellular kinetics in serous ovarian tumors, and the analysis of sAgNOR, mAgNOR and pAgNOR should be performed commonly. The prognostic significance of sAgNOR in patients with serous ovarian cancer was not proven.

Key words: nucleolar organizer regions, argyrophilic, ovarian tumors, serous.
Introduction

Neoplastic tumors arising from the surface epithelium of the ovary comprise a broad spectrum of neoplasms, ranging from serous to endometrioid, mucinous, transitional, clear cell and undifferentiated tumor types [1]. These histotypes have been recently associated with distinct molecular profiles, making it reasonable to conceive that the clinical outcome of molecular pathways may strongly affect the response to different drugs and the clinical outcome of patients [2, 3]. The most common primary ovarian cancer is of the serous histotype [1-3].

Many studies have been devoted to finding specific biomarkers in ovarian epithelial tumors and numerous features with varying degrees of accuracy have been described [4-7]. The analysis of the argyrophilic nucleolar organizer regions (AgNORs) is one of the methods [8]. Nucleolar organizer regions are segments of DNA that transcribe to ribosomal RNA and are situated on short arms of the acrocentric chromosomes 13, 14, 15, 21 and 22. The number of NORs is related to the cell cycle and the quantity of interphase NORs increases in cycling cells from the early G1 phase to the late S phase. In cancer tissues the number of NORs is closely related to both the percentage of cycling cells and S-phase cells [9, 10].

After silver staining, NORs can be easily identified as black dots exclusively situated throughout the nucleolar area, and are called AgNORs. NOR argyrophilia is due to a group of nucleolar proteins with a high affinity to silver. AgNOR analysis can be performed in three ways: I – the mean number of AgNORs per nucleus (mAgNOR), II – the mean percentage of nuclei with five or more AgNORs per nucleus (pAgNOR), III – the AgNOR area/nucleus ratio (sAgNOR) [11]. In our previous studies we analyzed the value of mAgNOR and pAgNOR but not sAgNOR in serous ovarian tumors [12-14].

The aim of the study was to assess: the value of sAgNOR analysis in ovarian serous epithelial adenomas, borderline tumors and cancers, the value of sAgNOR analysis in the prediction of disease-free survival (DFS) and overall survival (OS) in serous ovarian cancer.

Material and methods

The study included ovary tumors from 113 consecutive patients 17-83 years old (53.21 ±15.58) diagnosed and treated operatively for serous ovarian tumor (30 benign, 14 borderline and 69 cancers) in the Madurowicz Memorial Hospital of Lodz during 1998-2002. Detailed clinical characteristics of the study group are presented in Table I.

The tissues from surgery were analyzed. Sections 4 µm thick were cut from tissue blocks, previously routinely fixed in 10% buffered formalin and embedded in paraffin. One section was stained with hematoxylin and eosin for histopathologic diagnosis. Another sec-
tion was stained according to the one-step AgNOR method described by Howell and Black [10] and Plo-
ton et al. [15]: specimens were incubated in a mix-
ture of one volume 2% gelatin in 1% formic acid to
two volumes 50% silver nitrate and then washed
ten times with deionized distilled water. Histologi-
cal morphometry was performed by means of an
image analysis system consisting of a PC equipped
with an optical mouse, a Ver 2000 card (frame grab-
ber, true color, real time), produced by ADDA Tech-
nologies (Taiwan), and a Panasonic color TV camera
(Japan), coupled to a Carl Zeiss Jenaval microscope
(Germany). This system was programmed (Multi-
Scan software produced by Computer Scanning
Systems, Poland) to calculate the surface area of
the structure whose perimeter was traced, and the
total number of objects (semi-automatic function).

Both the counting of AgNORs and the morpho-
metric assessment were performed at 400× magni-
fication. AgNORs were seen as black or dark brown
dots within the nucleus. The following parameters
were estimated in 100 randomly chosen nuclei: (1)
nuclear area and nuclear outline (the outer limit of
a nuclear membrane was traced using the cursor of
an optical mouse), (2) the number of AgNORs per
nuclear area (these objects were automatically
counted and then followed with manual correction,
as needed). The randomization was made by the
operator. From these data the analysis of sAgNOR
was conducted. The sAgNOR was correlated with
tumor type and in cancers additionally with his-
torical factors included in the analysis were age at
diagnosis, clinical FIGO staging, radicality of surgery
(1. radical: lack of residual tumor, 2. optimal cytore-
duction: ≤ 1.0 cm diameter of residual tumor, 3. sub-
optimal cytoreduction: > 1.0 cm diameter of residual
tumor) and histological grading. Disease-free sur-
vival was defined as the period from primary surgery
until relapse. OS was defined as the period from
primary surgery until death or until complete fol-
low-up.

Statistical analysis

All data were analyzed using CSS Statistica soft-
ware (StatSoft Inc., Tulsa, OK, USA). Student’s t-test,
as a statistical method, was used to define differ-
ences between mean values of sAgNOR in serous
ovarian adenomas, borderline tumors and G1-3 can-
cers in stages I + II and III + IV and to compare the
mean values of age at diagnosis. The χ² test and
Fisher’s test were used to compare the history of
cancer, number of pregnancies and menopausal
status of patients. Spearman’s rank correlation was
used to correlate the sAgNOR and patients’ age,
tumor type, grading and FIGO staging. Kaplan and
Meier survival curves were calculated using univa-
riate survival analysis. The log-rank test was used
to compare survival curves by obtaining a χ² value.
A value of p less than 0.05 was considered signifi-

| Parameter         | n   | %   | DFS Hazard ratio | DFS 95% Confidence interval | Value of p | OS Hazard ratio | OS 95% Confidence interval | Value of p |
|-------------------|-----|-----|------------------|------------------------------|------------|----------------|------------------------------|------------|
| Age [years]       |     |     |                  |                              |            |                |                              |            |
| ≤ 50 (A)          | 14  | 26.92| 1.235            | 0.448                        | 0.135      | 0.5307         | 0.111                        | 0.0294     |
| 51-70 (B)         | 21  | 40.40| 1.134            | 0.651                        | 0.134      | 1.92           | 2.41                         | 0.685      |
| > 70 (C)          | 17  | 32.68| 1.134            | 0.815                        | 0.134      | 1.98           | 2.44                         | 0.685      |
|                   |     |     |                  |                              |            |                |                              |            |
| Grading           |     |     |                  |                              |            |                |                              |            |
| G1                | 13  | 25.00| 1.134            | 0.651                        | 0.134      | 1.92           | 2.41                         | 0.685      |
| G2                | 15  | 28.84| 1.134            | 0.751                        | 0.134      | 1.98           | 2.44                         | 0.685      |
| G3                | 24  | 46.16| 1.134            | 0.810                        | 0.134      | 1.98           | 2.44                         | 0.685      |
| Staging           |     |     |                  |                              |            |                |                              |            |
| I                 | 9   | 17.31| 1.713            | 0.091                        | 0.173      | 2.44           | 2.93                         | 0.114      |
| II                | 5   | 9.62 | 1.713            | 0.091                        | 0.173      | 2.45           | 2.97                         | 0.114      |
| III               | 31  | 59.62| 1.713            | 0.091                        | 0.173      | 2.45           | 2.97                         | 0.114      |
| IV                | 7   | 13.45| 1.713            | 0.091                        | 0.173      | 2.45           | 2.97                         | 0.114      |
| Radicality of surgery* |     |     |                  |                              |            |                |                              |            |
| R                 | 16  | 30.77| 1.075            | 0.748                        | 0.1075     | 2.04           | 2.63                         | 0.689      |
| OC                | 30  | 57.69| 1.075            | 0.748                        | 0.1075     | 2.07           | 2.66                         | 0.689      |
| SoC               | 6   | 11.54| 1.075            | 0.748                        | 0.1075     | 2.36           | 3.14                         | 0.846      |

sAgNOR [AgNOR/cm²] 0.954 2.30 3.11 0.734 0.954 2.36 3.14 0.846

*R – radical, OC – optimal cytoreduction, SoC – suboptimal cytoreduction
cant. A multivariate proportional hazard model (Cox) was used to test the prognostic value of features.

Results

The analysis of sAgNOR in ovaries from 113 patients treated operatively for serous ovarian tumor

A correlation between age and sAgNOR in patients with serous ovarian adenomas ($\delta = 0.177$; $p = 0.351$), borderline tumors ($\delta = 0.059$; $p = 0.840$) and cancers ($\delta = 0.169$; $p = 0.167$) was not found. Lower sAgNOR was found in benign adenomas than in cancers ($p = 0.008$) but not when compared to sAgNOR in borderline tumors ($p = 0.267$). A significant relationship in sAgNOR between borderline tumors and cancers was not found ($p = 0.103$) (Figure 1).

In G1 cancers sAgNOR was higher than in G3 cancers ($p = 0.029$) but not when compared to sAgNOR in G2 cancers ($p = 0.714$). A significant relationship in sAgNOR between G2 and G3 cancers was not found ($p = 0.118$) (Figure 2). sAgNOR in early staged (FIGO stages I-II) and advanced (FIGO stages III-IV) serous ovarian cancer was similar ($p = 0.539$). A correlation between sAgNOR and staging was not proven ($\delta = 0.028$; $p = 0.820$). The statistical analysis of sAgNOR as a function of tumor type, histological grading and clinical staging is presented in Table II. Figures 3-5 show the AgNORs in serous ovarian adenoma, borderline tumor and G3 cancer, respectively.

The analysis of survival as function of sAgNOR, age of patients, grading, FIGO staging and radicality of surgery in 52 patients with serous ovarian cancer

The follow-up period was 2-143 months (44.6 ±43.4). The DFS rate was 15.4%, and the OS rate was 21.2%. In univariate analysis only staging negatively correlated with better DFS and OS ($p = 0.016$, $p = 0.020$ respectively). sAgNOR ($p = 0.065$ – Figure 6; $p = 0.109$ – Figure 7), age of patients ($p = 0.102$; $p = 0.158$), grading ($p = 0.167$; $p = 0.120$) and radicality of surgery ($p = 0.156$; $p = 0.066$) were not associated with DFS or OS. In multivariate analysis no correlations of DFS or OS with sAgNOR, age of patients, grading, FIGO staging or radicality of surgery were proven (Table II).

Discussion

Silver staining is an easy and quick technique that can be performed on formalin-fixed paraffin-embedded sections, which enables accurate assessment of the changes in AgNOR patterns in tumor cell nuclei, and might provide new information on tumor biology [9-11, 15, 16]. The relationship between AgNOR count, age and performance status of the patients is not proven. It is known that in more rapidly proliferating tumors, AgNORs become disaggregated within the nucleus and nucleolus and the number of AgNORs increases [11]. It is postulated that in benign tumors of different origin, the mAgNOR value varies between one and two, and an increased mAgNOR value positively correlates with the number of acrocentric chromosomes, increased amount of DNA and aneuploidy [17]. An mAgNOR value larger than three is thought to be a characteristic marker of malignant tumors [11, 18].

The role of the AgNOR method in gynecological oncology was proven [8], and some reports describing the value of AgNOR assessment in epithelial ovarian tumors exist in the literature as well [7, 12-14, 16, 19-28]. Unfortunately, most of the studies were conducted in groups with heterogeneous histotypes of ovarian cancers, despite the distinct molecular profiles and the clinical outcome of patients, and only a few reports on homogeneous groups of ovarian epithelial neoplasms have been published [12-14, 16, 19-23].

The results of our previous studies in a homogeneous group of 113 ovarian serous epithelial tumors are similar to those published by Stemberger-Papić et al. (59 cases: 20 benign, 19 borderline and 20 cancers) [19] and confirmed that in ovarian serous epi-
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The total number of AgNORs increases from benign to borderline and malignant neoplasms [7, 13]. In the homogeneous group of ovarian mucinous epithelial tumors Versa-Ostojić et al. (46 cases: 16 benign, 15 borderline and 15 cancers) [20] and Ter-

| Characteristics of tumors | n   | %    | sAgNOR [AgNOR/cm²] | Statistical analysis |
|---------------------------|-----|------|--------------------|---------------------|
|                           |     |      | Min                | Max                 | Median  | SD       | Parameters | Value of p |
| Adenomas (A)              | 30  | 26.55| 0.38 × 10⁶         | 1.52 × 10⁶          | 0.73 × 10⁶ | 0.23 × 10⁶ | A-B       | 0.267      |
| Borderline tumors (B)     | 14  | 12.39| 0.56 × 10⁶         | 1.13 × 10⁶          | 0.81 × 10⁶ | 0.18 × 10⁶ | A-C       | 0.008*     |
| Cancers (C)               | 69  | 61.06| 0.41 × 10⁶         | 2.40 × 10⁶          | 0.96 × 10⁶ | 0.33 × 10⁶ | B-C       | 0.103      |
| Grading:                  |     |      |                    |                     |         |          |           |            |
| G1                        | 31  | 44.93| 0.66 × 10⁶         | 2.28 × 10⁶          | 1.02 × 10⁶ | 0.32 × 10⁶ | G1-G2     | 0.714      |
| G2                        | 21  | 30.43| 0.66 × 10⁶         | 2.40 × 10⁶          | 0.98 × 10⁶ | 0.37 × 10⁶ | G1-G3     | 0.029*     |
| G3                        | 17  | 24.64| 0.41 × 10⁶         | 1.35 × 10⁶          | 0.82 × 10⁶ | 0.24 × 10⁶ | G2-G3     | 0.118      |
| Staging:                  |     |      |                    |                     |         |          |           |            |
| I + II (S1)               | 14  | 20.29| 0.41 × 10⁶         | 1.38 × 10⁶          | 0.91 × 10⁶ | 0.26 × 10⁶ | S1-S2     | 0.539      |
| III + IV (S2)             | 55  | 79.71| 0.51 × 10⁶         | 2.40 × 10⁶          | 0.97 × 10⁶ | 0.34 × 10⁶ |           |            |

*Statistical significance

Figure 3. AgNORs in ovarian serous epithelial adenoma, 1000× magnification. After silver staining, NORs can be easily identified as black dots exclusively situated throughout the nucleolar area, and are called AgNORs (arrows)

Figure 4. AgNORs in ovarian serous epithelial borderline tumor, 1000× magnification. The total number of AgNORs (arrows) per nucleus increases from benign adenomas to borderline tumors

Figure 5. AgNORs in G3 ovarian serous epithelial cancer, 1000× magnification. The total number of AgNORs (arrows) per nucleus significantly increases from G1 to G3 serous ovarian cancers

Figure 6. sAgNOR analysis and DFS – univariate analysis

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labeled by various techniques including silver staining, nuclear fast red staining, and fluorescent staining [21]. In our previous study, we confirmed a significant association between staging and mAgNOR and pAgNOR in serous ovarian cancer as well [13]. Our present results in the same group show that the third parameter of AgNOR analysis, sAgNOR, remains rather constant during the progression and dissemination of the disease.

The value of AgNORs to predict long-term survival in patients with primary ovarian cancer has not been clearly explained yet [14, 27, 28]. Our previous studies examined 39 patients with a short observation period from initial surgery to second-look laparotomy. We found higher mAgNOR and pAgNOR to be related to a better response to adjuvant chemotherapy [14]. It is noteworthy that early relapses occur in tumors with high proliferative activity after remission, and generally prognosis for these patients is worse compared to patients with lower proliferative activity. Analyzing long-term treatment results, Muso found the number of AgNORs significantly higher in 37 patients with progressive ovarian cancer of different histological types, despite postoperative chemotherapy, compared to a group of patients who had undergone successful treatment [28]. Similarly, Sah et al. observed high AgNOR counts in a group of 84 patients with progressive disease, recurrence or death from tumor [27]. Our material does not confirm the above-mentioned observations and we found only the FIGO staging to be a valuable indicator of survival in univariate analysis.

In conclusion, sAgNOR is not a sufficient parameter to precisely characterize cellular kinetics in serous ovarian tumors, and the analysis of sAgNOR, mAgNOR and pAgNOR should be performed commonly. The prognostic significance of sAgNOR analysis in patients with serous ovarian cancer was not proven, but due to the small number of patients further prospective studies in larger populations are needed to assess the potential prognostic value of AgNOR count in ovarian cancer patients.

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