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Cancer stem cell-related marker NANOG expression in ovarian serous tumors using Western blotting and immunohistochemistry: comparison of two techniques

N. K. Šuster[1], M. Meznaric[2], N. Škorja[2], I. Virant-Klun[3], I. Verdenik[4], Š. Smrkolj[1,2]

[1]Department of Gynecology, Division of Obstetrics and Gynecology, University Medical Centre Ljubljana, Ljubljana
[2]Faculty of Medicine, University of Ljubljana, Ljubljana
[3]Department of human reproduction, Division of Obstetrics and Gynecology, University Medical Centre Ljubljana, Ljubljana
[4]Research Unit, Department of Obstetrics and Gynecology, University Medical Centre Ljubljana, Ljubljana (Slovenia)

Summary

Purpose of Investigation: The objective of the study was to evaluate cancer stem cell-related marker NANOG expression in ovarian serous tumors using Western blotting (WB) and to compare WB results to immunohistochemical (IHC) results of NANOG expression in the same tumors. *Materials and Methods:* Of the 37 ovarian tumor samples obtained intraoperatively, diagnosis of ovarian serous tumors was established histopathologically in 17 cases. WB and IHC for NANOG was performed on the parallel portions of the same ovarian tumors in the latter cases. The IHC staining samples were made up of a NANOG positive and a NANOG-negative group. Pursuant to summation of signal intensity and positive cell occurrence, the authors additionally divided the NANOG-positive group into three subgroups. Correlation coefficient between NANOG WB and NANOG IHC results was calculated. *Results:* NANOG measured by means of WB was significantly higher in the IHC determined NANOG-positive group than in the NANOG-negative group (p = 0.003). Comparison of the amount of NANOG measured by WB and IHC scores of individual cases revealed substantial dispersion of WB results among the NANOG subgroups; the dispersion was largest when NANOG was IHC only slightly-positive. In the NANOG moderate- and strongly-positive subgroups, WB values were higher and more homogenously arranged. In all IHC determined NANOG-negative cases NANOG WB values were low, with low value variability among tumor samples. However, correlation between NANOG WB results and NANOG IHC scoring subgroups revealed statistical significance (r = 0.73, p = 0.001). *Conclusion:* By means of WB and IHC the authors demonstrated NANOG to be a potential marker of ovarian high-grade serous carcinoma. Further research on the correlation between NANOG WB expression and clinical parameters is needed.

Key words: Ovarian cancer; Cancer stem cell-related marker; NANOG; Western blotting; Immunohistochemistry.

Introduction

Cancer stem cells (CSCs) and CSC markers are attractive in terms of cancer research, offering new perspective on ovarian cancer diagnosis and treatment.

NANOG protein is a transcription factor which, along with transcription factors OCT4 and SOX2, plays an important role in pluripotency and self-renewal maintenance in undifferentiated embryonic stem cells [1-4]. NANOG is not only a stem cell marker, but also the activator of tumorigenic pathways and MDR gene expression, and tumor growth [5]. NANOG expression was detected in germ cell tumors and other tumors, including ovarian cancer [6-9]. Conversely, NANOG was not expressed in relation to normal mature organization [3].

Epithelial ovarian carcinoma studies report that NANOG expression using immunohistochemistry (IHC), might be associated with high-grade tumors, advanced clinical stage and poor prognoses [9-11]. Study groups in these publications were heterogeneous in terms of histological type, tumor grading system and stage of disease, which made comparison difficult and provided results of limited value.

In the authors' previous research [12] they analyzed NANOG expression in ovarian serous tumors using IHC and for the homogenous group of high-grade serous carcinoma (HGSC) correlated it with clinical parameters. They demonstrated that CSC-related marker NANOG expression detected by IHC is significantly associated with high grade morphology, but not with clinical parameters.

In the present study the authors evaluated NANOG expression within ovarian serous tumors not only by IHC but also by means of Western blotting (WB). WB is a technique widely used in molecular biology to detect specific pro-

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teins, characterize their molecular weight, and estimate relative amounts [13]. In comparison to IHC, WB represents a semi-quantitative method which not only confirms NANOG expression, but also informs us about its amount.

The purpose of the study was to explore NANOG presence by means of WB in ovarian serous tumors and to estimate the relevance of NANOG as a potential marker of ovarian high grade serous carcinoma.

Materials and Methods

The study included 37 patients who had been surgically treated pursuant to ovarian cancer suspicion at the Division of Gynecology and Obstetrics, University Medical Centre Ljubljana, Slovenia, between May 2016 and March 2017. Tumor portions were frozen in liquid nitrogen and processed for NANOG WB analysis; the remainder was fixed in formalin and embedded in paraffin according to standard protocol for histopathological analysis and NANOG IHC analysis.

According to WHO Classification of Tumors of Female Reproductive Organs [14], a gynecologic pathologist diagnosed ovarian serous tumors in 17 cases; in 14 cases of HGCS, one case was predominantly low-grade ovarian serous carcinoma (LGSC) with small amount of borderline tumor and two cases were mainly borderline serous tumor and to some extent serous carcinoma.

Patient's clinical data was retrieved from their medical records, and clinical parameters analyzed as follows: patient age at diagnosis, stage of disease according to International Federation of Gynecology and Obstetrics (FIGO) classification; presence of ascites, surgical procedure type (cytoreductive surgery or diagnostic laparoscopic adnexectomy), residual tumor after primary cytoreductive surgery and type of chemotherapy. Cytoreductive surgery was performed according to the European Society of Gynaecological Oncology Ovarian cancer surgery guidelines. Postoperative tumor residue presence was defined in terms of presence or absence of macroscopic lesions (R classification)[15]. Patients in whom ovarian cancer was, at the time of diagnosis, considered inoperable received neoadjuvant chemotherapy. In cases where ovarian tumors were considered operable, cytoreductive surgery was performed and patients were referred for adjuvant chemotherapy.

Frozen patients' tumor tissue samples, diagnosed as ovarian serous tumors, were sectioned on cryostat. 50-100 5-µm thick sections were collected in Eppendorf tubes depending on specimen size. Proteins were extracted by adding 100 µl of the Ripa buffer, 150 mM NaCl, 5 mMEDTA, 1% Triton X-100, 1% SDS, 25 mM Tris-HCl pH 7.6, to which protease and phosphatase inhibitor cocktail sets were subsequently added; the sections were vortexed and left on ice for ten minutes, followed by sonification for 30 seconds at a rate of 26 pulses (50% amplitude, half cycle). After additional incubation on ice for ten minutes, the homogenates were centrifuged at 4°C at 14,000 g for 15 minutes. Supernatants were collected and protein concentration determined using a BCA protein assay kit-reducing agent compatible. 4X *Laemmli* buffer was added to the supernatants, which were next sonificated at a rate of 15 pulses (50% amplitude, half cycle), then boiled at 99°C for two minutes before finally being stored at -80°C.

Proteins were separated utilising 12% SDS-PAGE on 1.5 mm thick mini gels in mini-protean tetra cells with 100 µg of protein loaded per lane. Proteins were transferred to 0.2 µm PVDF membranes using a transfer buffer with methanol at a constant voltage of 20V for one hour, followed by high intensity transfer of 1A constant for 30 minutes with cooling in a blotter. Unstained protein ladder standards ranging from 10 kD to 200 kD were used. After transfer, gels were stained using Coomassie blue and PVDF membranes with 0.2% Panceau S in 3% trichloroacetic acid to control transfer efficiency and for protein standard visualization.

Membranes were blocked in 7.5% non-fat dried milk and 0.2% BSA in PBS-0.1% Tween 20 for one hour at room temperature, washed in PBS-0.1% Tween 20, and incubated with primary antibodies: anti-NANOG rabbit monoclonal antibodies (1: 1000; anti-NANOG antibody overnight at 4°C. After washing in PBS-0.1% Tween 20, short blocking for 20 minutes with 5% non-fat dried milk was performed, followed by incubation with secondary antibodies, i.e. goat anti-rabbit IgG (H+L), horseradish peroxidase conjugated (1:5000) in 5% non-fat dried milk in PBS-0.1% Tween 20. After extensive washing in PBS-0.1% Tween 20, immunoreactive proteins were visualised using chemiluminescent substrate.

Autopsy-normal human brain and normal-menopausal ovary tissue, removed intraoperatively, served as NANOG-negative control, autopsy-normal human testicle tissue serving as NANOG-positive control. All tissues were cryopreserved. In addition, possible unspecified immunoreactive bands resulting from secondary-antibody cross-immunoreactivity were verified through the incubation of parallel lanes of ovarian serous carcinoma with secondary antibodies only. According to manufacturer instructions, a band of approximately 37 kD was considered NANOG specific.

Image analysis program was used for NANOG WB quantification, measuring the intensity and area, i.e. the amount, of the NANOG specific band.

IHC analysis was performed on tissue microarrays (TMA). Three representative areas from each tumor were marked on Hematoxylin and Eosin (H&E) slides. After retrieving the corresponding paraffin blocks, the marked areas were identified and sampled. Three 2-mm cores were taken from the selected areas of each tumor block and each biopsy specimen was placed in one of the three recipient paraffin blocks. To achieve better cylinder and recipient paraffin block adhesion, the constructed blocks had been incubated at 37˚C for three hours prior to placing. The first section of the TMA block was stained with H&E to verify arrayed tissue adequacy, consecutive sections were placed on salinized slides for IHC staining. An automatic slide stainer was used for IHC staining. After deparaffinisation, antigen retrieval utilizing CC1 Ventana reagent was facilitated for 48 minutes. Rabbit anti-Human NANOG monoclonal antibodies served as the primary antibody. Further to manual application of primary antibodies, the slides were incubated at a dilution of 1:25 for 30 minutes at 37˚C. An OptiView kit was used to detect antigen presence. Normal testicular tissue and testicular embryonal carcinoma served as positive controls, while elimination of the primary antibody and normal menopausal ovarian tissue served as negative controls. Positive NANOG staining was considered, if a brown reaction was detected in cells' nuclei and/or cytoplasm.

Intensity and stained tumor cell percentages were evaluated. Staining intensity was evaluated as: no staining (0), weak staining (1+), moderate staining (2+), and strong staining (3+). The mean value of all three sampling areas of individual tumor was calculated and results were placed in four score classes: 0, 1, 2, and 3. The percentage of positive cells in a tumor sample was then evaluated and the mean value of all three sampling areas of individual tumor was, once again, placed in four score classes: 0 (0-4%), 1 (5-49%), 2 (50-74%), and 3 (75-100%). IHC scores, the sum of intensity scoring and percentage scoring, ranged from zero to six.

In cases where IHC scores were zero, samples were placed in the NANOG-negative group (NANOG 0). In cases of IHC scores between one and six, samples were placed in the NANOG-positive group. Pursuant to IHC scoring, samples from NANOG-pos-

itive group were additionally divided into three subgroups: NANOG +1 (IHC score 1, 2), NANOG +2 (IHC score 3, 4), and NANOG +3 (IHC score 5, 6).

The Mann-Whitney test was used to test the difference between NANOG WB quantitative values in NANOG-positive and NANOG-negative groups classified by IHC. Spearman's correlation coefficient was calculated for WB quantitative values and IHC ordinal categorical variables. Results were set to be statistically significant at $p < 0.05$.Statistical analysis was performed using SPSS for Version 24.0.

Results

The mean age of patients was 62.7±10.2 (range, 48-81) years. Of the HGSC group, three (21.4%) patients were diagnosed in FIGO Stage I, one (14.3%) FIGO Stage II, and ten (71.4%) FIGO Stage III. Ascites was present in eight (57.1%) cases. In seven (50%) cases, cytoreductive surgery was performed; R0 resection being evidenced in five (71.4%) cases. Pursuant to cytoreductive surgery, patients were referred for adjuvant chemotherapy. The remaining seven (50%) cases resulted in bilateral adnexectomy performed due to inoperable disease presence, and patient referral for neoadjuvant chemotherapy. In cases of LGSC and borderline serous tumors with disease diagnosed as either FIGO Stages II or III, cytoreductive surgery was performed and patients received adjuvant chemotherapy.

The NANOG values in ovarian serous tumors measured by WB (in arbitrary units) ranged between 100 and 1,200 (Figures 1 and 2). The amount of NANOG in the positive control, testicle tissue, was around 300. All cases of ovarian tumor samples with values above 300 belonged to HGSC. The amount of NANOG in the negative control, brain tissue, was hardly detectable i.e. bellow 100. Normal ovarian tissue sample, both borderline serous tumors, and LGSC had NANOG values between 100 and 300. Enlarged WB images of selected ovarian serous tumor cases are presented in Figure 2.

Nine of the 17 tumors (52.9%) were NANOG-positive and histologically HGSC. LGSC and borderline serous tumors were NANOG-negative. Of all HGSCs, nine (64.3%) were NANOG-positive and five (35.7%) NANOG-negative. HGSC's samples were according to IHC staining scores represented in NANOG-positive subgroups as follows: four (28.6%) in NANOG +1; three (21.4%) in NANOG +2; and two (14.3%) in NANOG +3. In all NANOG-positive cases the protein was predominantly expressed in cell cytoplasm.

NANOG measured by means of WB was significantly higher in the IHC NANOG-positive group than in the NANOG-negative group ($p = 0.003$) (Figure 1). There was also statistically significant correlation between WB results and NANOG IHC scoring subgroups ($r = 0.73$, $p = 0.001$). Amount of NANOG measured by WB in comparison to IHC score (NANOG subgroup) of individual cases is presented in Figure 3.

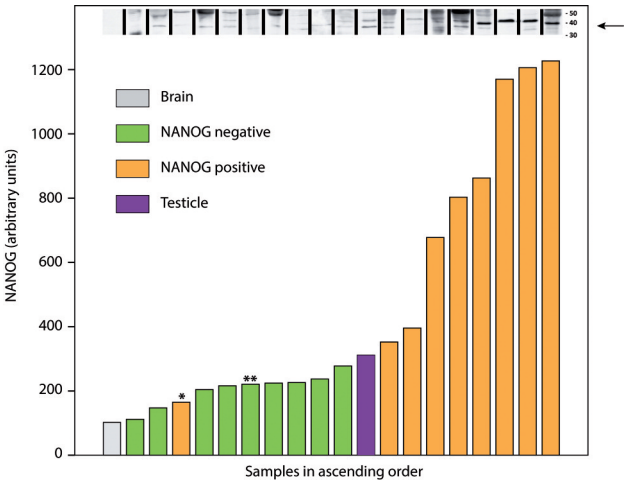

Figure 1. — Comparison of NANOG detection in ovarian serous tumors by means of Western blotting and immunohistochemistry. The ordinate shows the amount of NANOG detected by Western blotting in arbitrary units. Abscissa shows NANOG-positive cases in orange and NANOG-negative cases in green as classified by immunohistochemistry. The amount of NANOG in the positive control, testicle tissue, and negative control, brain tissue, are violet and grey respectively. All NANOG-negative cases have NANOG below 300, and the majority of NANOG-positive cases have NANOG above 300, except one (*) which has NANOG amount around 200. Normal ovary' (**) NANOG value is also around 200. The amount of NANOG in the negative control, brain tissue, is hardly detectable i.e. bellow 100. In the chart above, corresponding, individual-case Western blot images are demonstrated. A band of approximately 37 kD (arrow) is considered NANOG-specific.

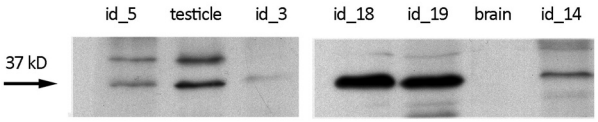

Figure 2. — Selected and enlarged Western blotting images of ovarian serous tumor. Left: Immunohistochemically NANOG-negative ovarian high grade serous carcinoma (id_5) and borderline serous tumor (id_3) cases; normal-testicle tissue serve as the positive control. Right: Immunohistochemically NANOG-positive ovarian high grade serous carcinomas (id_18, id_19 and id_14) cases, with brain tissue serve as negative control; bands of approximately 37 kD (arrow) were considered NANOG specific.

Discussion

To the best of the present authors knowledge, CSC-related marker NANOG in ovarian tumor tissue, has not been assessed in terms of WB thus far. NANOG presence in terms of WB was evidenced in different cells and cell lines [16, 17]; however, only a few studies have analyzed NANOG presence in animal and human tissue including reproductive organs, such as uteri and ovaries [18-20]. The

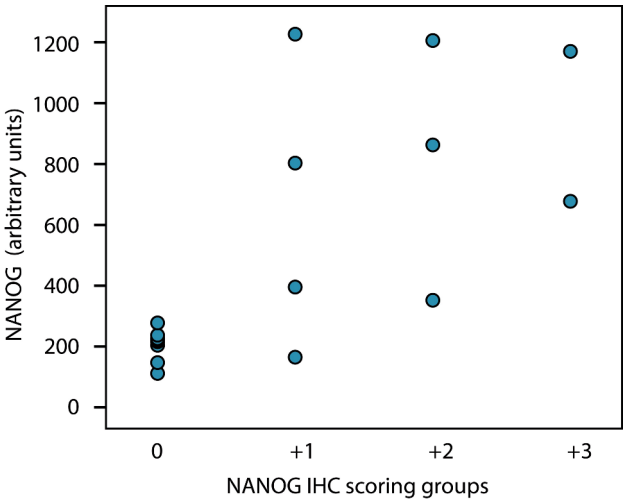

Figure 3. — NANOG measured by Western blotting (in arbitrary units) compared to individual immunohistochemical scores. Cases with an IHC score of zero (NANOG 0) have NANOG amounts below 400; cases with IHC scores 1 or 2 (NANOG +1) display the largest dispersion of NANOG amounts, ranging from 200 to 1,200; cases with IHC scores 3 or 4 (NANOG +2) have values around and above 400; both cases of IHC scores 5 or 6 (NANOG +3) have values higher than 600.

present study is the first to assess NANOG expression by means of WB in ovarian tumor tissue.

All cases of ovarian serous tumor samples expressing WB NANOG above positive control (normal human testicle) belonged to HGSC. LGSC, both borderline serous tumors and normal ovarian tissue had WB NANOG values below positive control. Similarly, NANOG was IHC positively expressed *only* in tumor samples of HGSC. The latter is also in accordance with results of the previous work [12]. Both, WB and IHC results suggest NANOG to be a potential marker of ovarian HGSC.

There was only one case in which WB and IHC results did not match. In this case, WB NANOG values were low, but IHC NANOG expression was positive, although only slightly. This mismatch might be the consequence of different sampling areas of tumor tissue used for WB and IHC. On the other hand, in three combined ovarian serous tumor cases in which uneven outcomes of IHC and WB would be expected, as different parts of tumors consisted of different histologic types, the results were similar for both techniques. These ovarian tumors were to different degree composed of borderline serous and LGSC tumor tissue. According to results of our previous study IHC NANOG expression was in all cases of borderline serous tumors and LGSCs NANOG-negative [12]. Considering latter data, sampling from different regions of tumor, which should be NANOG-negative, would not change the result of NANOG quantification. Never the less, tumors' histologic variability in case of NANOG-positive tumor might matter.

Despite significant correlation between WB and IHC in the present study, comparing the amount of NANOG measured by WB to IHC scores of individual cases revealed the considerable dispersion of results in some NANOG subgroups. The dispersion was the largest when NANOG was only slightly-positive (NANOG +1). In the IHC NANOG moderate- and strongly-positive group (NANOG +2 and +3) WB values were, in general, higher and more homogenously arranged. In all IHC NANOG negative cases, WB values were low and with low value variability among tumor samples. As in some cases, it was not possible to predict the amount of WB NANOG from the IHC NANOG, correlation between WB NANOG and clinical parameters in a larger group of patients would be beneficial.

IHC and WB have their advantages and disadvantages. In both methods, synthetic or animal-derived antibodies are used to react with a specific target protein (antigen). IHC combines immunological technique with microscopic anatomy to visualize the distribution and localization of a specific antigen within cells in their proper histological context. WB, on the other hand, is a semi-quantitative method in which the amount of the immunochemically detected protein can be quantified by densitometry [13]. If quantitative data is desired, ELISA or mass spectroscopy should be employed. Nevertheless, WB results are, in either case, expressed in numbers, so comparison of samples is relatively easy. Interpretation of IHC results, on the other hand, might be more subjective, particularly in unclear cases, which might have occurred in the present study in cases of slightly-positive NANOG expression; therefore besides experience, overall IHC standardization is needed [21].

NANOG protein contains a highly conserved region of 60 amino acids, termed home-box domain, and N- and C-terminal domains required for its proper nuclear localization [22]. Its middle region on the other hand holds a potent nuclear export motif which allows NANOG to move in and out of the nucleus [23]. Cytoplasmatic and nuclear localization of NANOG detected by IHC was reported in ovarian, cervical, and breast cancer [7, 9, 10, 12, 24]. Also in the current study NANOG was IHC *predominantly* documented in cell cytoplasm. NANOG cytoplasmatic presence in cancer tissue might be related to the transcriptional regulation of cytoplasm mitochondrial DNA [9].WB of total tissue homogenates, as used in this study, does not inform about the subcellular localization of antigens, so the present authors suggest that future studies using WB for NANOG detection should analyze NANOG in terms of subcellular fractions and results correlate with clinical parameters.

Despite the limitation in this study associated with a relatively small number of patients and challenge in terms of methodology facilitation in case of WB performing on ovarian tumor tissue, the present results contribute to the limited body of knowledge concerning ovarian serous cancer stem cells identification.

Conclusion

By means of WB and IHC the present authors have proved NANOG to be a potential marker of ovarian HGSC. In the further studies on WB NANOG expression in HGSC the analyses of NANOG subcellular fractions should be done and correlation with clinical parameters determined.

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Corresponding Author:
Š. SMRKOLJ, M.D., PHD
Department of Obstetrics and Gynecology
University Medical Centre Ljubljana
Šlajmerjeva 3
SI-1000 Ljubljana (Slovenia)
e-mail: spsmrkolj@gmail.com