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Nucleolin and nucleophosmin expression in seminomas and non-seminomatous testicular tumors

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Abstract

Introduction. Testicular tumors are heterogeneous group of neoplasms divided mainly into two types: seminomas and non-seminomas. Nucleolin (NCL) and nucleophosmin (NPM) are abundant nucleolar proteins involved in many physiologic and pathologic processes including cancer. Their overexpression was found in many tumors but it was not studied in testicular cancer.

Material and methods. The study was performed on tissue microarrays of 19 seminomas, 21 embryonal carcinomas and 11 yolk sac tumors. The expression of NCL and NPM was detected with monoclonal antibodies and visualized with EnVision FLEX/HRP technique. Immunohistochemical reactions were measured with Aperio ImageScope Software and analyzed as means of percentages of all immunopositive cells in three groups of reaction intensity, *i.e.* 3+, 2+, and 1+ as well as of H-score.

Results. Seminomas showed higher expression of nucleolin indicated by higher H-score and higher percentage of positive cells than non-seminomas. The differences in subpopulations of NCL-positive cells were also found. Embryonal carcinomas and yolk sac tumors showed lower expression of NCL than seminomas indicated by H-score. The percentage of NCL-positive cells did not differ between embryonal carcinomas and seminomas while there were significant differences in subpopulations of cells. The percentage of NCL-positive cells in yolk sac tumors was lower than in seminomas. The results show different heterogeneity of subpopulations of NCL-positive cells in embryonal carcinomas and yolk sac tumors compared to seminomas. The analysis of nucleolin expression in embryonal carcinomas and yolk sac tumors showed no differences between these two tumor types. No differences in nucleophosmin expression between seminomas and non-seminomas were found.

Conclusions. The differences in the expression of nucleolin between two groups of germ cell testicular tumors found in the current study indicate a new aspect of biology of these neoplasms and require further studies on the role of nucleolin in germ cell tumorigenesis. *(Folia Histochemica et Cytobiologica 2019, Vol. 57, No. 3, 139–145)*

Key words: nucleolin; nucleophosmin; germ cell tumor; seminoma; testis; IHC

Introduction

Testicular tumors are the most common cancer in young men between puberty and forties in Europe and they account for 1–3% of all cancers in men. The incidence and death rate in Poland are among the highest in European countries [1]. Germ cell testicular tumors are heterogeneous group of neoplasms and based on their diverse histology and biological behavior they can be divided into seminomas and non-seminomas. Median age of patients with seminoma is 35 years and ones with non-seminomas is 25 years [2]. Compared to non-seminomas, seminomas present relatively uniform histology with large cells containing regular nuclei with one or more nucleoli [3]. Non-seminomas is diverse group of neoplasms that among others include embryonal carcinoma and yolk sac tumors as a pure malignancies or elements of mixed germ cell tumors. Embryonal carcinomas vary in histologic presentation from sheets of primitive-appearing cells to glandular or papillary structures with highly pleomorphic atypical nuclei with many nucleoli

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and frequent mitoses. Less common in adult men yolk sac tumors show numerous histologic patterns from solid through microcystic and glandular to classic one with Schiller-Duval bodies [3, 4]. The most common histologic and molecular precursor of seminomas and non-seminomas is germ cell neoplasia *in situ* (GCNIS), which arises from primordial germ cells that failed to differentiate into spermatogonia. One of the first events in neoplastic transformation of germ cells is an expression of Oct4. Different programming of GCNIS results in transformation to seminoma or to embryonal carcinoma that is a neoplastic counterpart of the human embryonal stem cell [5–7]. GCNIS express several stem cell related markers such as Oct4, NANOG, c-KIT, PLAP, TSPY [2, 6, 8]. Seminoma cells have limited capacity to differentiate while embryonal carcinoma cells can differentiate into embryonic somatic lines or extraembryonal and trophoblastic structures [8]. Both embryonal carcinoma cells and seminoma cells express Oct4 and NANOG. Difference between seminomas and non-seminomas include expression of Sox17, high hTERT expression and high telomerase activity in seminomas and Sox2 expression in embryonal carcinomas [2, 9, 10].

Nucleolus seen within the nucleus in standard histologic staining with hematoxylin and eosin is a structure composed of three elements: fibrillar centers (FC), dense fibrillar components (DFC) and granular components (GC) and it is a site of ribosome biogenesis [11]. Nucleolin (NCL), highly conservative protein, is a three-domain structure involved in many processes such as nucleolus formation, transcription of rDNA, maturation of rRNA, ribosomal assembly and nucleocytoplasmic transport, regulation of apoptosis and cell differentiation [11–13]. NCL is involved in wide variety of pathologic processes including cancer (as a promotor or suppressor), inflammation, neurodegeneration [12]. This protein is mainly detected in nucleolus but also in nucleoplasm outside nucleolus [11, 14], cytoplasm and cell membrane [12]. Nucleophosmin (NPM) is another abundant nucleolar protein with possible aberrant cytoplasmic localization. Similarly to NCL it shuttles between nucleolus and nucleoplasm. Both proteins function as chaperones and they interact with numerous protein partners including themselves [15]. NCL and NPM overexpression was found in many tumors [16, 17]; however, their expression in testicular tumors has yet not been analyzed.

Materials and methods

The study was approved by the Bioethical Commission of the Pomeranian Medical University in Szczecin, Poland (approval number KB-0012/267/09/18).

All cases of germ cell testicular tumors diagnosed in Departments of Pathology of the 1st and the 2nd Clinical Hospitals of Pomeranian Medical University, Szczecin, Poland and Multispecialty Hospital, Gorzów Wielkopolski, Poland between May 2004 and April 2018 were re-analyzed by one pathologist (MM) and representative slides and paraffin-embedded tissue blocks were selected for constructing tissue microarrays (TMA). All tissue samples for routine pathologic diagnosis were formalin-fixed and paraffin embedded. Two main tumor types were selected for further studies — pure seminomas and mixed germ cell tumors. Due to high heterogeneity of the latter ones, cases containing only embryonal carcinomas and yolk sac tumor elements were selected for the study. 19 cases of seminomas and 27 cases of mixed germ cell tumors (21 cases of embryonal carcinoma elements and 11 cases of yolk sac tumor elements) were included in the study.

Immunohistochemistry. All immunohistochemical (IHC) studies were performed on tissue microarray (TMA) slides. During histological evaluation areas of interest were encircled on a glass slide and afterwards cores of tissue were taken from representative sites of original paraffin blocks and were inserted into recipient paraffin blocks. To ensure the representativeness of the material 3 cores were taken from each area of interest. Each TMA were cut into 3 μm-thick section, deparrafinized and antigens were retrieved for IHC reactions (PT Link, Dako, Glostrup, Denmark). Endogenous peroxidase was blocked with hydrogen peroxide and slides were incubated with primary antibody. Two mouse anti-human antibodies were used for detecting studied proteins — anti-nucleolin (monoclonal antibody; clone 4E2; Abcam, plc., Cambridge, UK; 1:2000 dilution, 37°C for 90 min) and anti-nucleophosmin (monoclonal antibody; clone FC82291; Abcam, plc.; 1:2500 dilution, 37°C for 90 min). Antigens retrieval procedures were tested and antibodies were titrated for optimal IHC reactions that were visualized with EnVision FLEX/HRP (Dako). Slides were counterstained with hematoxylin, dehydrated and sealed with coverslips. One section of each TMA was also stained with hematoxylin and eosin for microscopic control of cores quality. Slides were immediately scanned with ScanScope slide scanner (Aperio Technologies Inc., Vista, CA, USA) and the analyses of IHC reactions were performed with Aperio ImageScope software (Aperio Technologies Inc.) (Fig. 1). Each evaluated area was selected manually to avoid analysis of necrotic areas or non-neoplastic tissues encountered in cores. Results of the analysis were measured and calculated by the software and were presented in three forms: a) mean percentage of all positive cells, b) percentage of cells with different expression of proteins [+3 (highly positive), +2 (medium positive), +1 (low positive) and 0 (negative)] and c) H-score, which was calculated with following formula: *H-score = (% of +3-positive cells × 3) + (% of +2-positive cells × 2) + (% of +1-positive cells)*.

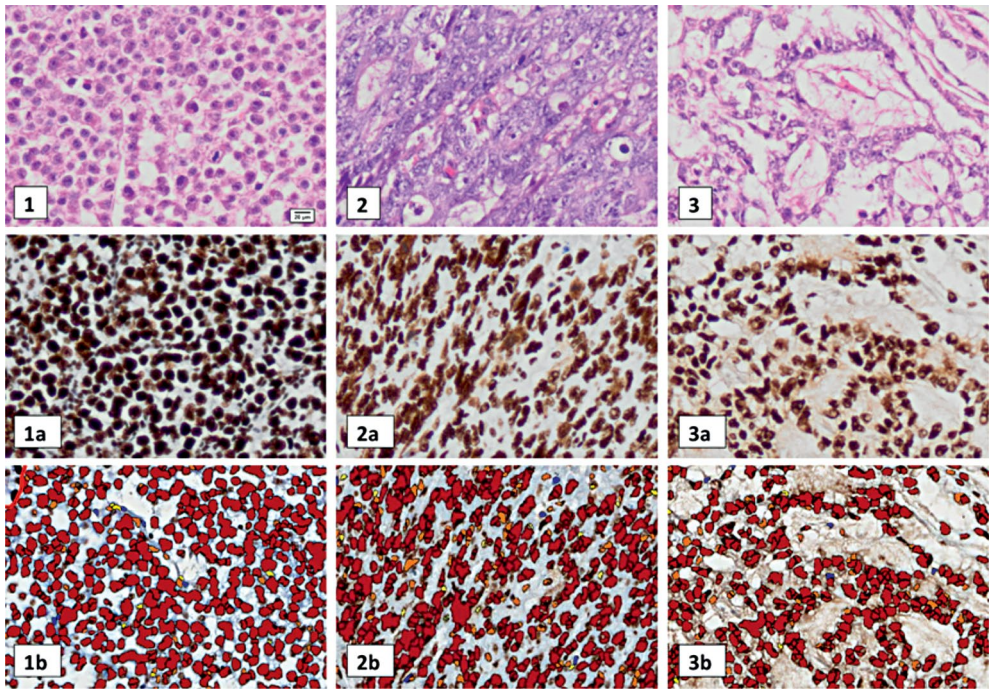

Figure 1. Nucleolin (NCL) expression in seminoma, embryonal carcinoma and yolk sac tumor. **1–3** hematoxylin and eosin staining; **1** — seminoma, **2** — embryonal carcinoma, **3** — yolk sac tumor. Microphotographs **1a–3a** present NCL immunoreactivity, whereas figures **1b–3b** present analyses of the NCL immunoreactivity by Aperio Scan Scope software in respective tumors. Sections were stained by immunohistochemistry as described in Materials and Methods.

Statistical analysis. Data distribution was tested with Shapiro-Wilks test. Some data showed non-Gaussian distribution thus Kruskal-Wallis test was used for finding differences between more than two groups and subsequently U-Mann-Whitney test for differences between two groups. All analyses were performed with STATISTICA for Windows 13.1 (StatSoft, Kraków, Poland).

Results

The mean age of patients with testicular tumors analyzed was 35.5 ± 12.3 years (mean ± SD) while mean tumor size was 35.5 ± 19.1 mm. Detailed data is presented in Table 1.

Patients with seminomas were older than patients with non-seminomas but the difference did not reach statistical significance ($p = 0.07$). When compared between separate groups of non-seminomas, patients with embryonal carcinoma component were significantly younger than patients with seminoma ($p = 0.008$) and patients with yolk sac tumor component ($p = 0.03$). There were no age differences between patients with yolk sac tumor component and patients with seminoma ($p = 0.86$). Tumor size showed no statistically significant differences between any groups studied. Mean number of nuclei measured in each case was 4941 ± 1874 (mean ± SD).

Nucleolin expression and subpopulation of NCL--positive cells in seminomas and non-seminomas

We analyzed the percentage of NCL-positive cells, percentages of subgroups of highly positive (+3), medium positive (+2), low positive (+1) and negative cells (0) as well as H-score in seminomas and non-seminomas. Seminomas showed higher expression of NCL indicated by higher H-score and higher percentage of positive cells than non-seminomas ($p = 0.0009$ and $p = 0.024$, respectively). Interesting results were found in subpopulations of NCL-positive cells. The percentage of +3-positive cells was higher ($p = 0.0004$) while percentages of +2, +1 and negative cells were lower in seminomas than in non-seminomas ($p = 0.001$, $p = 0.002$ and $p = 0.025$, respectively) (Table 2). Differences in percentages of subgroups of NCL-positive cells indicate the heterogeneity of expression of nucleolin between seminomas and non-seminomas.

Nucleolin expression and subpopulation of NCL-positive cells in seminomas vs embryonal carcinoma- and yolk sac tumor-component of non-seminomas

We compared NCL expression between seminomas and two subtypes of non-seminomatous tumors — embryonal carcinomas and yolk sac tumors separately. Both

Table 1. Patients' age and tumor size

	Seminomas	Non-seminomas	Embryonal carcinomas	Yolk sac tumors
Age (years)	38.6 ± 12.0[a]	32.4 ± 10.4	28.6 ± 7.0	39.3 ± 12.2[b]
Tumor size [mm]	36.7 ± 25.4	34.8 ± 14.3	31.7 ± 11.5	44.0 ± 19.2

The results present mean ± SD. Letters by superscripts indicate significant differences between groups: [a]p = 0.008, seminomas *vs.* embryonal carcinomas; [b]p = 0.03, yolk sac tumors *vs*. embryonal carcinomas.

Table 2. Semiquantitative analysis of the immunohistochemical expression of nucleolin in seminomas and non-seminomas of the testis

Immunoreactivity	Seminomas	Non-seminomas	p
% of positive nuclei	97.71 ± 1.6	95.88 ± 3.80	0.024
% of +3 positive nuclei	81.41 ± 7.67	68.87 ± 12.94	0.0004
% of +2 positive nuclei	13.29 ± 5.10	21.41 ± 8.80	0.001
% of +1 positive nuclei	3.01 ± 1.67	5.56 ± 3.28	0.002
% of negative nuclei	2.27 ± 1.60	4.11 ± 3.81	0.025
H-score	273.81 ± 11.94	255.03 ± 20.74	0.0009

The results present mean ± SD. +3, +2 and +1 denote highly, medium, and low positive immunoreactivity, respectively. H-score was calculated as described in Material and Methods.

Table 3. Semiquantitative analysis of the immunohistochemical expression of nucleolin in seminomas, embryonal carcinomas and yolk sac tumors of the testis

Immunoreactivity	Seminomas	Embryonal Carcinomas	Yolk Sac Tumors
% of positive nuclei	97.71 ± 1.6[a]	95.97 ± 4.45	95.70 ± 2.24
% of +3 positive nuclei	81.41 ± 7.67[b, c]	68.80 ± 11.59	69.00 ± 15.83
% of +2 positive nuclei	13.29 ± 5.10[d]	21.80 ± 7.44	20.72 ± 11.35
% of +1 positive nuclei	3.01 ± 1.67[e, f]	5.34 ± 3.00	5.98 ± 3.87
% of negative nuclei	2.27 ± 1.60[g]	4.02 ± 4.46	4.30 ± 2.23
H-score	273.81 ± 11.94[h, i]	255.35 ± 20.72	254.42 ± 21.79

Table legend as for Table 2. Letters in superscripts indicate significant differences between groups: [a]p = 0.015 seminomas *vs* yolk sac tumors; [b, c]p = 0.0002 and p = 0.049, seminomas *vs.* embryonal carcinomas and seminomas *vs.* yolk sac tumors, respectively; [d]p = 0.0005 seminomas *vs.* embryonal carcinomas; [e, f]p = 0.003 and p = 0.045, seminomas *vs* embryonal carcinomas and seminomas *vs.* yolk sac tumors, respectively; [g]p = 0.01 seminomas *vs* yolk sac tumors; [h, i]p = 0.0016 and p = 0.02, seminomas *vs.* embryonal carcinomas and seminomas *vs.* yolk sac tumors, respectively.

embryonal carcinomas and yolk sac tumors showed lower expression of NCL than seminomas indicated by H-score (p = 0.0016 and p = 0.02, respectively).

The percentage of NCL-positive cells in embryonal carcinomas did not differ from the percentage of NCL-positive in seminomas while there were significant differences in subpopulations of cells. We found lower population of +3-positive cells (p = 0.0002) and higher populations of +2-positive and +1-positive cells in embryonal carcinomas than in seminomas (p = 0.0005 and p = 0.003, respectively) (Table 3).

The percentage of NCL-positive cells in yolk sac tumors was lower than in seminomas (p = 0.015). The differences in subpopulation of cells in yolks sac tumors were slightly different than in embryonal carcinomas. We found lower population of +3-positive cells (p = 0.049) and higher populations of +1-positive cells (p = 0.045) and negative cells (p = 0.01) in yolk sac tumors than in seminomas but we found no differences in +2-positive cell subpopulation (Table 3).

The results show different heterogeneity of subpopulations of NCL-positive cells in embryonal carcinomas and yolk sac tumors compared to seminomas.

The analysis of NCL expression in embryonal carcinomas and yolk sac tumors showed no significant differences between these two tumor types either in H-score value or in percentages of respective groups of NCL-positive and negative cells (Table 3).

Table 4. Parameters of immunohistochemical expression of NPM in germ cell testicular tumors

Immunoreactivity	Seminomas	Non-seminomas	p
% of positive nuclei	89.89 ± 7.62	83.03 ± 17.29	0.32
% of +3 positive nuclei	42.87 ± 22.00	36.19 ± 23.85	0.35
% of +2 positive nuclei	30.33 ± 7.43	30.15 ± 10.33	0.94
% of +1 positive nuclei	16.19 ± 10.17	16.71 ± 9.42	0.75
% of negative nuclei	10.60 ± 7.23	16.97 ± 17.29	0.45
H-score	205.48 ± 45.82	185.56 ± 63.22	0.35

Table legend as for Table 2.

Nucleophosmin expression and subpopulation of NPM-positive cells in seminomas and non-seminomas

We also analyzed the percentage of NPM-positive cells, percentages of subgroups of highly positive (+3), medium positive (+2), low positive (+1) and negative cells (0) as well as H-score in seminomas and non-seminomas. We found no significant differences in any analyzed parameters for NPM expression between seminomas and non-seminomatous group (Table 4). The expression of NPM did not differ between embryonal carcinomas and yolk sac tumors (data not shown).

Discussion

In the current study, we analyzed nuclear expression of two proteins — nucleolin and nucleophosmin in seminomas and non-seminomas by standard IHC technique performed on tissue microarrays. Only NCL showed differences of expression between these tumor types. Nuclear expression of NCL was significantly higher in seminomas than in embryonal carcinomas and yolk sac tumors, while there were no statistically significant differences between the latter ones. We found no differences in NPM expression between tumor types studied. There were no previous studies on these two proteins in testicular pathology including germ cell tumors.

NCL and NPM are among most abundant nucleolar proteins forming argyrophilic nucleolar organizer regions (AgNOR) that were extensively studied since eighties in different pathologies but only few publications on AgNOR in testicular pathology can be found. Meng *et al.* analyzed area of AgNOR in testicular carcinoma *in situ* (CIS) finding higher level of AgNOR in CIS associated with non-seminomas than with seminomas. They also mentioned that mean area of AgNOR in solid tumor cells did not differ between seminomas and non-seminomas but they pointed to difficulties in objective assessing the parameters studied [18]. Ohyama *et al.* studied forty-five patients with invasive testicular tumors and they found higher number of AgNOR per nucleus in seminomas than in non-seminomas [19]. Results of the study by Ohyama *et al.* are in concordance with the results of our current study. It may be stated that higher AgNOR parameters in seminomas are related to higher NCL expression since it is one of the main AgNOR-related proteins [11, 14].

The expression of NCL in different histologic types of cancer was previously studied in limited series of tumor types. The results of our previous study on a group of 87 ductal and 11 lobular invasive breast cancers showed higher expression of NCL in nucleolus and in karyoplasm in ductal than lobular breast cancers [17]. Xu *et al.* showed higher expression of nucleolin in squamous cell lung cancers than in pulmonary adenocarcinomas but their results were not statistically significant [20].

At the molecular level seminomas differ from non-seminomas by many markers and pathways including proteins, miRNA, mRNA and DNA methylation [21]. Seminomas molecular characteristic mirrors early germ cells while embryonal carcinoma resembles embryonic stem cells [8]. Among markers of pluripotency, Sox2 is the one that is expressed in non-seminomas with no expression in seminomas [9, 10]. Experiments on glioblastoma stem-like cells showed that increased NCL expression downregulated Sox2. NCL decreased stem-like characteristics of Sox2 expression and the inhibitory effect of NCL was due to the transcription inhibition and also was observed on protein level. NCL knockout increased the expression of Sox2. Lower expression of NCL was accompanied by the upregulation of glioblastoma stem cell (GSC) markers including Sox2 [22]. The higher nucleolin expression in Sox2- negative seminomas may suggest similar molecular pathway to the one described in glioblastoma stem-like cells.

Nuclolin is a protein interacting with human telomerase reverse transcriptase (hTERT) and this

interaction is necessary for nucleolar localization of hTERT and its activity [23]. NCL overexpression causes nucleolar localization of telomerase in cancer cells [24]. Schrader *et al.* found high expression and high activity of hTERT in both seminomas and embryonal carcinomas (with high range of results) but lower in yolk sac tumors; however the differences between all groups studied were not statistically significant [25]. Turnbull *et al.* found some variants of SNPs in *hTERT* gene independently associated with testicular germ cell tumors, one of them showing stronger association with seminomas than with non-seminomas [26]. The differences in NCL expression between seminomas and non-seminomas found in our study may suggest the possible different interactions of nucleolin with hTERT in testicular tumors that might be modified by single nucleotide polymorphism in *hTERT* gene.

We did not find any differences in nucleophosmin expression between seminomas and non-seminomas. Published results on NPM expression in different histologic tumor types including malignant and benign ones are ambiguous. Pianta *et al.* studied 46 thyroid tumors (10 benign and 33 malignant) and they found higher NPM expression in papillary cancers than in follicular or undifferentiated cancers. On the other hand, NPM expression in benign follicular adenomas was higher than in follicular or undifferentiated carcinomas [27]. Sari *et al.* studied NPM expression in 68 renal tumors (9 benign and 59 malignant) and found nuclear NPM expression in chromophobe, papillary and clear cell cancers with no expression in benign oncocytomas and highly aggressive sarcomatoid cancers. They also found higher nucleolar NPM expression in benign oncocytomas and in highly aggressive sarcomatoid renal cell cancers than in clear cell or papillary renal cell cancers [28]. These results may suggest that NPM expression is neither related to benign *vs* malignant tumors nor to low *vs*. high malignant cancers. Relatively high standard deviation of NPM expression found in current study indicates high heterogeneity of NPM expression in all tumor types. Further studies are required for assessing the role of NPM in solid tumor pathology.

In this study, the expression of NCL and NPM in seminomas and non-seminomas as well as different subpopulations of cells expressing both proteins were analyzed for the first time. The differences in subpopulations of cells expressing NCL between seminomas and non-seminomas indicate new aspect of the biology of these tumors. However, our results should be considered as initial observations that require further studies including correlations with clinical data, also prospective ones, for the studied patients (*e.g.* disease-free survival). Moreover, the number of yolk sac tumors cases should be increased for clinical studies. Overall, our results show differences in the expression of selected nucleolar protein in testicular tumors that require further studies.

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Conflict of interest

Authors declare no conflict of interest.

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