

THE STUDY OF PHOSPHATE TRANSPORTER NAPI2B EXPRESSION IN DIFFERENT HISTOLOGICAL TYPES OF EPITHELIAL OVARIAN CANCER

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The identification of markers that are specifically expressed by different histological types of epithelial ovarian cancer (EOC) may lead to the development of novel and more specific diagnostic and therapeutic strategies. Sodium-dependent phosphate transporter NaPi2b (or MX35 ovarian cancer antigen) is a novel perspective marker of EOC. To date, the studies on NaPi2b/MX35 expression in different histological types of EOC are limited. **Aim:** To examine NaPi2b/MX35 expression in different histological types of epithelial ovarian tumors. **Methods:** Here, we describe the analysis of NaPi2b expression in serous (n = 17), endometrioid (n = 8), and mucinous ovarian tumors (n = 3) by Western-blotting (WB), immunohistochemistry and RT-PCR. **Results:** The results of immunohistochemical and WB analysis showed that benign and well-differentiated malignant papillary serous tumors as well as well-differentiated malignant endometrioid tumors overexpress NaPi2b protein. However, no overexpression of NaPi2b was detected in benign and malignant mucinous tumors as well as in poorly differentiated endometrioid tumors. Notably, the expression NaPi2b mRNA was detected in all investigated histological types of EOC. **Conclusion:** We have shown the differential expression profile of NaPi2b phosphate transporter at protein level in various histological types of epithelial ovarian cancer. This finding might facilitate the development of more effective approaches for diagnosis and treatment of this disease.

Key Words: epithelial ovarian cancer, tumor markers, phosphate transporter NaPi2b.

Ovarian cancer is the most common cause of death from cancers of the female genital tract [1–4]. The high mortality rate results in part from the frequent diagnosis of ovarian cancer at advanced stages. Epithelial tumors of ovary comprise 58% of all ovarian neoplasms and more than 90% of malignant tumors of ovary [5]. EOC arises from ovarian surface epithelium (OSE) that has "uncommitted" phenotype and retains the capacity to differentiate into different types of cells in response to environmental signals [6]. During ovarian carcinogenesis, the epithelium of ovary could differentiate into fallopian tube epithelium (papillary serous tumors), endometrial epithelium (endometrioid tumors), colonic or endocervical epithelium (mucinous tumors) and component of endometriosis (clear cell tumors). The histological analysis of EOC indicate that papillary serous tumors represent 50–60% of all ovarian cancers, and the remaining tumors exhibit endometrioid (25%), mucinous (4%) and clear cell (4%) histology [7]. It is obvious that the observed tumor heterogeneity has a molecular basis, and the identification of molecular markers specific for different histological types of epithelial ovarian cancer can lead to the development of more effective treatment approaches.

Recently, studies from a collaborative consortium have led to the identification of sodium-dependent phosphate transporter NaPi2b as ovarian cancer antigen, termed MX35 [8]. The identity of MX35 antigen was confirmed by screening of ovarian cancer cell line OVCAR3 cDNA expression library with monoclonal antibody MX35 and by affinity purification of MX35 antigen followed by mass spectrometry. MX35 antigen was originally identified with the use of monoclonal antibody MX35, obtained from mice immunized with fresh ovarian carcinoma cells and selected by extensive analysis of normal and malignant tissues and cell lines. Biochemical and immunohistochemical studies revealed that MX35 MAb recognizes a cell surface glycoprotein with molecular weight of about 95 kDa, which is overexpressed in 90% ovarian cancer specimens but shows restricted expression in normal tissues. Clinical studies with Fab fragments of radiolabeled MX35 antibody suggest a therapeutic potential in patients with ovarian cancer [9, 10].

The human sodium-dependent phosphate transporter NaPi2b is encoded by *SLC34A2* gene, and belongs to the type II family of sodium-dependent phosphate transporters (SLC34 family). It is involved in maintaining the homeostasis of inorganic phosphate in human body by regulating intestinal Pi absorption [11]. In normal tissues, the expression of NaPi2b at the protein level is restricted to small intestine [12], lung [13], liver [14], mammary and salivary glands [15, 16]. The overexpression of NaPi2b transporter was revealed in epithelial ovarian carcinomas by SAGE analysis and real-time RT-PCR [17]. However, there are

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Abbreviations used: EOC – epithelial ovarian cancer; IH – immunohistochemistry; OSE – ovarian surface epithelium; SLC34A2 – solute carrier family 34 (sodium phosphate), member 2; WB – Western-blotting.

almost no data concerning the expression of NaPi2b/MX35 protein by different histological types of EOC. The investigation of NaPi2b expression by different histological types of EOC might provide the insight on its prognostic value and the potential for developing immunotherapeutic approaches in ovarian cancer.

In this study, we compared the expression of NaPi2b protein between normal ovarian tissues and different histological types of EOC, such as serous, endometrioid and mucinous ovarian tumors.

MATERIALS AND METHODS

Tissue samples. Tumor samples were obtained from ovarian cancer patients ($n = 28$) admitted for tumor resection at the National Cancer Institute (Kyiv, Ukraine). The types of EOC were confirmed by histopathological examination at the Department of Pathology, National Cancer Institute (Kyiv, Ukraine). Normal ovarian tissue samples ($n = 10$), obtained during surgical treatment of the patients with endometrial cancer (mean age 46 years, range 19–69 years), were used as the control. The mean age of patients with ovarian cancer 47 years (range 22–69 years). The study was approved by the Ethics Committee of the Institute of Molecular Biology and Genetics, and consent forms were obtained from all patients.

Western-blot analysis. We have analyzed NaPi2b expression in 28 ovarian cancer samples and 10 normal ovarian tissue samples. Tissues samples were homogenized in RIPA buffer (20 mM TrisHCl, pH 7.5, 0.150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.1% SDS) supplemented with PMSF (Sigma, Steinheim, Germany), and Protease Inhibitor Cocktail (Sigma, Steinheim, Germany) and then centrifugated at 4 °C for 30 min. Soluble fractions of lysates (50 µg per sample) were separated by 8% SDS-PAGE at non-reducing conditions. Separated proteins were transferred to a PVDF membrane for 2 h at 80 V (Perkin Elmer, Boston, MA) using a wet transfer cell (Pharmacia Biotech). After the transfer, the membrane was blocked in PBST buffer (1 x PBS with 0.05 % Tween), containing 3% BSA and then incubated with anti-NaPi2b (0.5 mg/ml) mAb [18] at 4 °C overnight. After extensive washing in PBST buffer, the membrane was incubated with anti-mouse IgG secondary antibody (1 : 5000) for 1 h (Promega, Madison, WI). The immune complexes were detected by ECL system (Amersham, Uppsala, Sweden). GAPDH was used as a loading control.

Immunohistochemistry. Immunohistochemical analysis of ovarian cancer samples with anti-NaPi2b MAbs was performed according to a standard protocol. Briefly, representative sections of ovarian tumors were prepared from paraffin blocks. Endogenous peroxidase was quenched with H₂O₂ (3%) in 0.01% PBS. After blocking of non-specific binding with avidin-biotin blocking solution (Vector Laboratories, Burlingame, CA, USA), tissue sections were incubated overnight at 4 °C with anti-NaPi2b mAb (10 µg/ml). Then, sections were incubated with biotinylated secondary antibodies for 2 h at room temperature at 1 : 400 dilution

(goat anti-mouse biotinylated IgG, Sigma, Steinheim, Germany), followed by incubation with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA) for 30 min at RT and developed with diaminobenzidine solution. Haematoxylin was used for counterstaining. Standard microscopy was performed using a Zeiss Universal microscope (Zeiss, Jena, Germany), and images were captured using digital Axiocam software.

Total RNA isolation and RT-PCR analysis. Total RNA was isolated by acid guanidinium-thiocyanate-phenol-chloroform extraction procedure [19]. Briefly, 1 ml of denaturation solution (4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7.0, 5% sarcosyl, 0.1 M 2-mercaptoethanol) was added to 100 mg of homogenized tissues. Sequentially, 0.1 ml of 2 M sodium acetate, pH 4, 1 ml of phenol (water saturated), and 0.2 ml of chloroform-isoamyl alcohol mixture (49 : 1) were added to the homogenate. After centrifugation at 10 000 g for 20 min at 4 °C, total RNA was precipitated from aqueous phase with the same volume of isopropanol at –20 °C for 1 h. The pellet was resuspended in 75% ethanol, air dried and dissolved in 50 µl nuclease-free water.

Total RNA (5 µg) was converted to cDNA with M-MuLV Reverse Transcriptase (Fermentas) at 37 °C for 60 min using oligo dT primers. Produced cDNA (100 ng) were amplified using following primers — forward GTCATCACTAAGCCCTTCA and reverse CAGGCAACCACAGAGGAC — for 30 cycles in 50 µl total volume of PCR buffer 5 x PCR (Fermentas) containing 10 mM dNTP, one unit Taq polymerase, 20 pmol of each primer. The amplification was performed in DNA Thermal cycler (Perkin Elmer) under following conditions: 94 °C for 60 s, 60 °C for 30 s and 72 °C for 60 s. Amplified products were separated on a 1% agarose gel and visualised.

RESULTS

In this study, we have examined NaPi2b expression in ovarian tumors of serous, endometrioid and mucinous histology. In a panel of 28 ovarian tumors, there were 17 serous tumors (3-cystadenomas, 1 papillary cystadenoma and 13 papillary carcinomas); 8 endometrioid tumors (1 cystadenoma, 4 well-differentiated and 3 poor-differentiated carcinomas); and 3 mucinous tumors (1 cystadenoma, and 2 carcinomas) (Table). The techniques of Western blot, immunohistochemistry and RT-PCR were employed to examine the expression profile of NaPi2b in these tumors and to compare them with normal ovarian tissues.

Table. The characterization of analyzed ovarian cancer samples

Histological type of EOC	Number of malignant samples (including type of differentiation)	Number of benign samples
Serous Papillary	13, w/d	1
Non-papillary	–	3
Endometrioid	4, w/d 3, p/d	1
Mucinous	2, n/a	1

Notes: w/d – well-differentiated; p/d – poor-differentiated; n/a – not available.

Immunoblot analysis of NaPi2b expression in ovarian samples was performed at non-reduced conditions using monoclonal antibody raised against the extracellular

loop of human NaPi2b (188–360aa). The specificity of anti-NaPi2b MAb was tested with the use of recombinant NaPi2b expressed in bacteria as GST fusion protein (Fig. 1, a). Furthermore, using WB we have confirmed the expression of endogenous NaPi2b protein in ovarian cancer cell line (OVCAR3), kidney carcinoma cell line (SKRC18) that are MX35 positive cell lines, and HEK293 stably expressing NaPi2b protein (Fig. 1, b).

It has been previously demonstrated that NaPi2b protein is recognized on WB as a panel of immunoreactive bands with different molecular weights, reflecting the state of the protein glycosylation [12–16, 20–22]. The results presented on Fig. 1, c show that NaPi2b is recognized in ovarian tumor tissue lysates as a band with molecular weight of 100 kDa. Notably, only some ovarian tumors overexpress NaPi2b.

Further analysis has indicated that the expression of NaPi2b is detected in all (13/13) investigated samples of papillary serous tumors. In the case of benign serous tumors, NaPi2b expression was detected only in papillary cystadenoma (1/1), whereas serous cystadenomas with non-papillary structure did not express NaPi2b (0/3) (see Fig. 1, c).

In endometrioid tumors NaPi2b expression was found in some of tested tumors. Interestingly, well-differentiated endometrioid carcinomas (4/4) were characterized by overexpression of NaPi2b, while poor-differentiated endometrioid carcinomas (0/3) and endometrioid cystadenoma (0/1) did not express detectable level of NaPi2b (see Fig. 1, c). The expression of NaPi2b protein in WB was not detected in mucinous cystadenoma (0/1) and mucinous carcinomas (0/2) as well as in control ovarian lysates (0/10) (see Fig. 1, c).

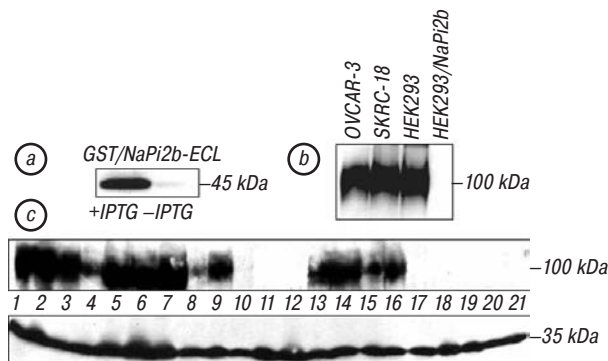


Fig. 1. Western-blot analysis of NaPi2b protein with the use of anti-NaPi2b MAb generated against extracellular loop (ECL) of NaPi2b. a, Expression of GST/NaPi2b-ECL in bacteria was induced by 1 mM IPTG. Total lysates were resolved by SDS-PAGE and probed with anti-NaPi2b MAb. b, Endogenous expression of NaPi2b protein in OVCAR-3, SKRC-18 cell lines and HEK293 cells stably expressing NaPi2b. c, Expression of NaPi2b in different types of ovarian cancer and normal ovary (upper part), lane 1–8 — papillary serous carcinoma, lane 9 — papillary serous cystadenoma, lane 10 — serous cystadenoma, lane 11 — endometrioid cystadenoma, lane 12 — poor-differentiated endometrioid carcinoma, lane 13–16 — well-differentiated endometrioid cystadenoma, lane 17 — mucinous cystadenoma, 18 — mucinous carcinoma, lane 19–21 — normal ovary. GAPDH was used as a loading control (lower part)

We performed immunohistochemical (IH) detection of NaPi2b in all samples of ovarian tumors mentioned above and normal ovarian tissues. Using IH staining the presence of NaPi2b was detected only on the apical

surface of the epithelial cells that is a characteristic feature of NaPi2b expression.

We found that the surface epithelium of normal ovary as well as ovarian stroma were negative for NaPi2b staining (Fig. 2, a). IH analysis of serous tumors showed strong staining with anti-NaPi2b MAb in papillary serous carcinomas (Fig. 2, b) and papillary serous cystadenomas (Fig. 2, c) but no staining was detected in serous cystadenomas with non-papillary structures (Fig. 2, d). Moreover, well-differentiated endometrioid tumors exhibited strong staining with the use of NaPi2b MAb (Fig. 2, e), which was absent in poor-differentiated and benign endometrioid tumors. All examined cases of benign and malignant mucinous ovarian tumors (3) were negative for NaPi2b (Fig. 2, f). The results presented above indicate a good correlation between the expression of NaPi2b protein and the histological types of tumors. Differential pattern of NaPi2b protein expression in various types of ovarian tumors prompted us to examine NaPi2b protein expression in normal tissues of female reproductive system, such as fallopian tubes, endometrium and endocervix. We observed the positive staining with NaPi2b MAb in normal fallopian tube epithelium (Fig. 3, a) and endometrium (Fig. 3, b) but not in endocervical epithelium (Fig. 3, c).

Total RNA derived from 28 ovarian cancer tissues and 10 normal ovarian tissues were investigated by RT-PCR analysis with the use of *SLC34A2* specific primers (see Materials and Methods), which were designed for unique sequences of *SLC34A2* gene. The product of amplification (350 bp) was analysed on an agarose gel. The results presented in Fig. 4, a have shown *SLC34A2* mRNA expression in all tissues analyzed.

DISCUSSION

We have recently identified sodium-dependent phosphate cotransporter NaPi2b as MX35 antigen, which is known to be overexpressed in 90% of human ovarian cancers [8, 9]. The pattern of NaPi2b expression in normal tissues and ovarian cancer makes NaPi2b/MX35 antigen a potential target for the development of immunotherapeutic and diagnostic approaches of EOC.

NaPi2b is a transmembrane glycoprotein that possesses 8 transmembrane domains, a large extracellular loop (188–360aa) with several potential sites of glycosylation, and the N- and C-terminal cytoplasmic tails facing the cytosol [8]. In addition to MX35 antibody, we have recently developed several monoclonal antibodies recognizing the extracellular loop of human NaPi2b [18]. To our knowledge, the expression of NaPi2b/MX35 protein in different histological types of EOC have not been analysed so far. The elucidation of NaPi2b expression profile in various types of ovarian cancer might be useful not only for understanding molecular mechanisms of carcinogenesis, but also for the verification of diagnosis, prognosis and treatment strategies.

In this study, we have analyzed NaPi2b expression at protein and mRNA levels in different types of epithelial ovarian cancer and normal ovarian tissues. We

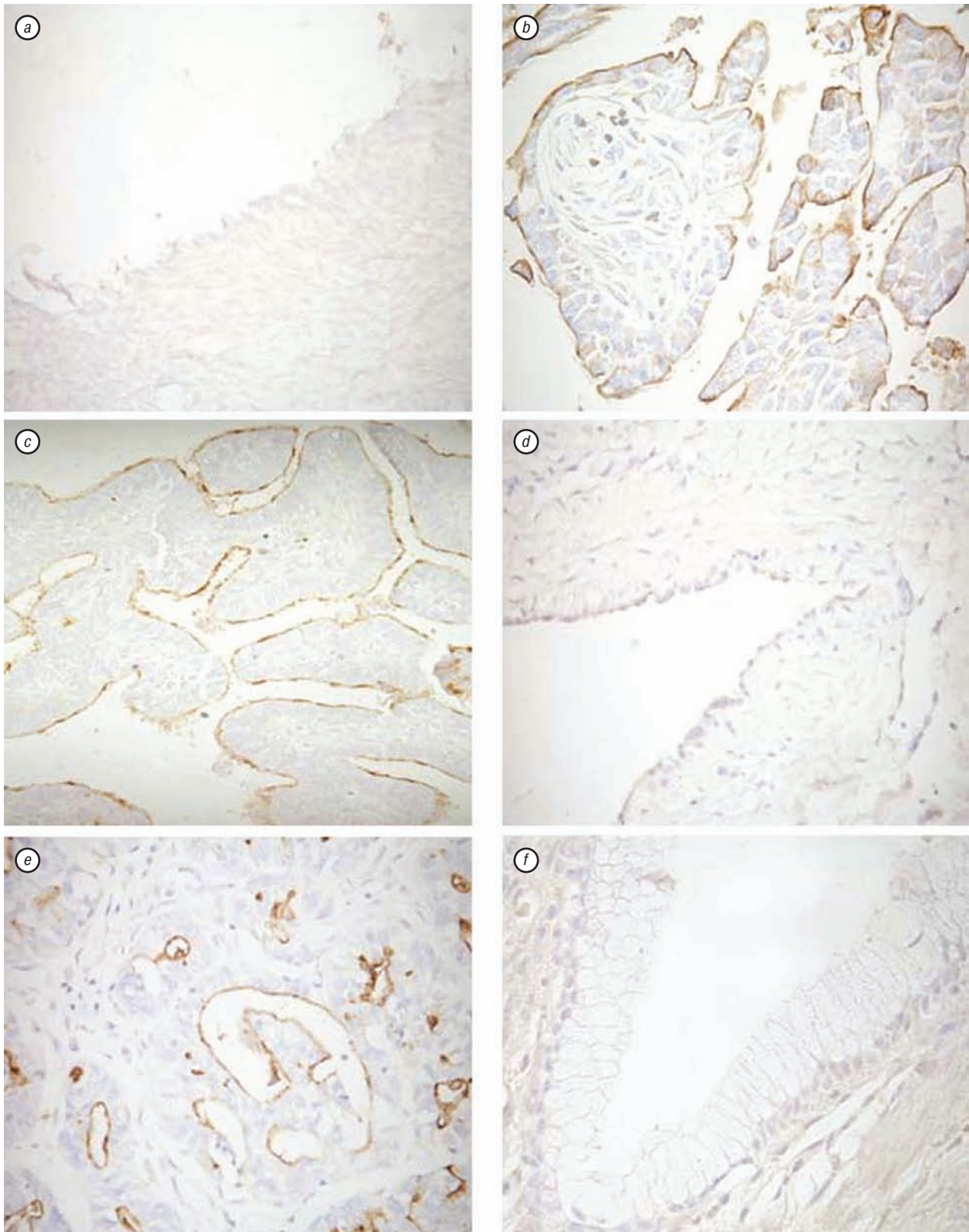


Fig. 2. Immunohistochemistry of ovarian tumors and normal ovaries samples with the use of anti-NaPi2b MAbs. *a*, normal ovary; *b*, papillary serous carcinoma; *c*, papillary serous cystadenoma; *d*, serous cystadenoma; *e*, well-differentiated endometrioid carcinoma; *f*, mucinous cystadenoma

have observed a good correlation of NaPi2b protein expression detected by WB and IH in ovarian tumor samples of different histological types. Our results showed that NaPi2b protein is overexpressed in papillary serous tumors and low-grade endometrioid tumors when compared to mucinous ovarian cancer. The most common histological type of EOC is papillary

serous carcinomas, which are often associated with concentric rings of calcification known as psammoma bodies [23]. Notably, breast and papillary thyroid cancers, which are characterized by aberrant expression of NaPi2b, are also affected by calcifications [24, 25]. It has been demonstrated that the downregulation of the NaPi2b transport function results in the deposition

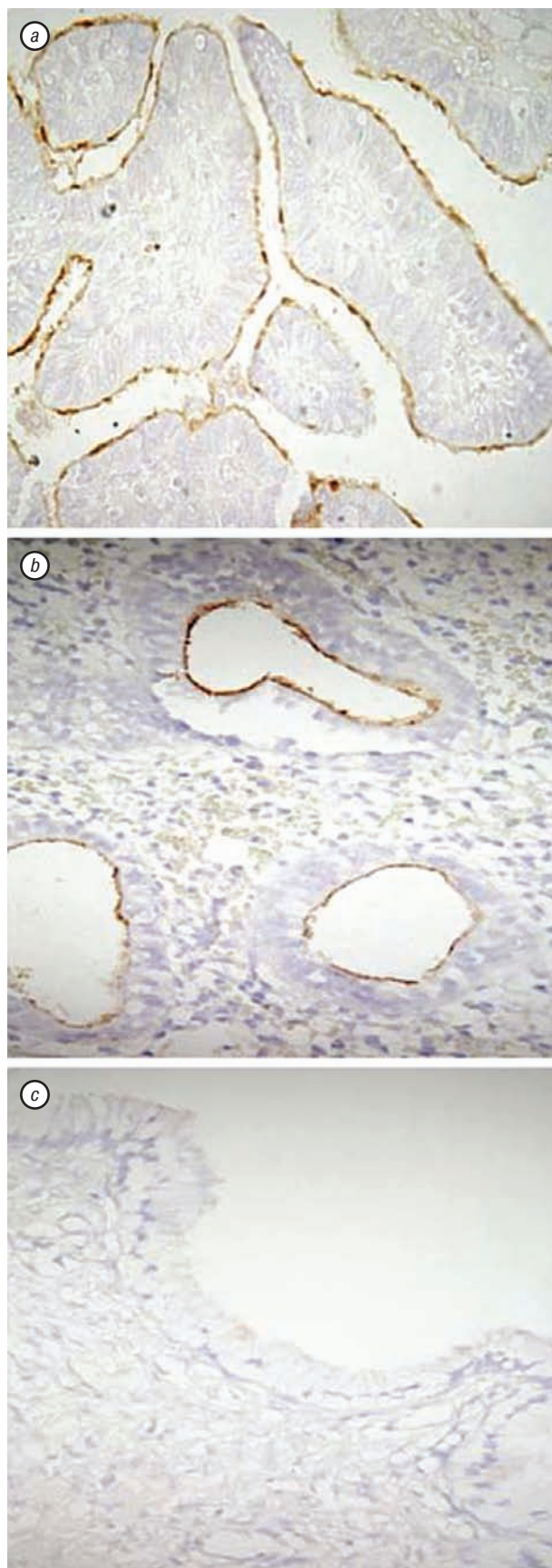


Fig. 3. Immunohistochemistry of normal fallopian tubes (a), endometrium (b), endocervical epithelium (c) with anti-NaPi2b mAb of calcium phosphate microliths in patient’s lungs. This phenomenon is caused by mutations in *SLC34A2* gene of phosphate transporter, which finally manifests by itself the development of pulmonary alveolar micro-

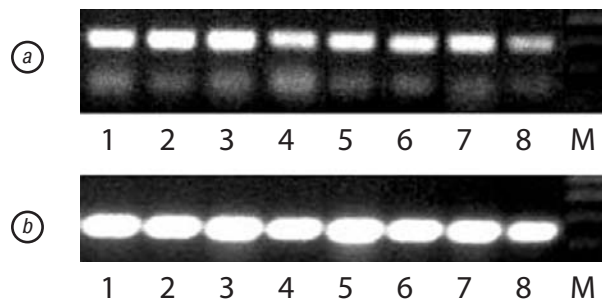


Fig. 4. RT-PCR analysis of ovarian tumor samples with *SLC34A2* primers (a) and primers to beta-actin (b). Lane 1 — papillary serous carcinoma, lane 2 — papillary serous cystadenoma, lane 3 — serous cystadenoma, lane 4 — endometrioid cystadenoma, lane 5 — well-differentiated endometrioid carcinoma, lane 6 — mucinous cystadenoma, lane 7 — mucinous carcinoma, lane 8 — normal ovary lithiasis (PAM) [26]. Based on these data we propose that the calcification in papillary serous ovarian cancer could be associated with the failure of calcium phosphate homeostasis due to the aberrant expression or mutations in NaPi2b phosphate transporter gene.

Since low tumor grade has been associated with good outcome and survival [27, 28], the overexpression of NaPi2b in well-differentiated papillary serous and endometrioid carcinomas could be a marker of a good prognosis. Our data correlate well with the results published by Rangel *et al.* [17], showing that well differentiated epithelial ovarian tumors tend to express higher level of NaPi2b.

IH analysis of normal tissues of female reproductive system with NaPi2b MAb revealed its expression in fallopian tube epithelium and endometrium, but not in endocervical epithelium. Since ovarian surface epithelium of papillary serous tumors resembles epithelium of fallopian tube, and endometrioid tumors — endometrial epithelium, and mucinous tumors — colonic or endocervical epithelium [29], it is not surprisingly that only papillary serous and endometrioid tumor of low grade overexpressed NaPi2b protein. So, the overexpression of NaPi2b protein in well-differentiated papillary serous and endometrioid tumors is linked to differentiation of ovarian surface epithelium into fallopian tubes epithelium and endometrium, respectively.

The expression of NaPi2b mRNA in different types of EOC showed no correlation with that of NaPi2b protein detected by IH and WB. We observed mRNA NaPi2b expression in all investigated histological types of EOC and did not reveal significant difference between samples with high and low level of NaPi2b protein expression. The absence of correlation between NaPi2b mRNA and protein expression could indicate the regulation of NaPi2b expression at the level of translation that should be further investigated. Our data concerning NaPi2b mRNA expression correlate with the data reported by Rangel *et al.* [17], showing up-regulation of *SLC34A2* expression in serous, endometrioid, mucinous and clear-cell tumors of EOC by real-time RT-PCR analysis.

In summary, our data demonstrate that phosphate transporter NaPi2b is overexpressed in well-differentiated papillary serous and endometrioid ovarian tu-

mors. Moreover, we found that differential expression of NaPi2b in EOC may be the consequence of changes in ovarian epithelium differentiation during malignant process. The findings of this study might facilitate the rational development of new diagnostic modalities and targeted therapies of ovarian malignancies.

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