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Creation of cellular models for the analysis of sodium-dependent phosphate transporter NaPi2b, a potential marker for ovarian cancer

V. S. Gryshkova, D. S. Lituyev, V. V. Filonenko, R. G. Kiyamova

Institute of Molecular Biology abd Genetics, National Academy of Sciences of Ukraine
150 Academician Zabolotnogo str., Kyiv, Ukraine, 03680

v.v.filonenko@imbg.org.ua

The aim of present study was to develop a model for a functional analysis of a recently identified marker of the ovarian cancer – sodium-dependent phosphate transporter NaPi2b. For this purpose, we have created HEK293 stable cell lines expressing wild type or mutant forms of NaPi2b (T330V substitution in a large extracellular loop and a 6 amino acid residues deletion in the C-terminal cytoplasmic tail), revealed in the ovarian cancer cell lines. The expression of wild type and mutant forms NaPi2b in the stable cell lines created was confirmed by Western-blot analysis with monoclonal antibodies against NaPi2b. The cellular models described here will be useful for studying the function of sodium-dependent phosphate transporter NaPi2b in health and disease.

Keywords: sodium-dependent phosphate transporter NaPi2b, mutation, anti-NaPi2b MAb.

Introduction. Epithelial ovarian cancer (EOC) is one of the leading causes of cancer-related death in women and the leading cause of gynecologic cancer death. The lack of specific markers for EOC makes it difficult to achieve the clinical objective for early detection and therapy. Thus, the identification and characterization of novel ovarian cancer markers is crucial for the development of novel diagnostic and immunotherapeutic approaches in gynecologic oncology and for understanding the molecular mechanisms of malignant growth.

The recent findings suggest that the sodium-dependent phosphate transporter NaPi2b could be considered as a potential prospective marker of ovarian cancer. Firstly, NaPi2b is overexpressed in ovarian cancer in comparison to normal tissues and other types of cancer [1]. Secondly, NaPi2b was recently identified as MX35 cancer antigen by two independent approaches: a) screening of ovarian cancer cell line OVCAR3 cDNA expression library with monoclonal antibody MX35; and b) affinity purification of MX35 antigen followed by mass spectrometry analysis [2]. MX35 MAb was generated more than 20 years ago at Memorial Sloan-Kettering Cancer Center by immunizing mice with fresh ovarian carcinoma cells and screening generated hybridomas with a panel of ovarian cancer cell lines [3].

Further studies showed that MX35 antigen is expressed at high level inapproximately 90 % of human ovarian epithelial cancers, which created the base for using humanized MX35 MAb in early phase clinical trials [3, 4]. In normal tissues, the expression of sodium-dependent phosphate transporter 2b at the protein level is restricted to small intestine [5], lung [6], liver [7], mammary and salivary glands [8, 9].

The human sodium-dependent phosphate transporter NaPi2b is encoded by *SLC34A2* gene which be-

Oligonucleotide primers used for cloning and site-directed mutagenesis

Transporter	Primer
FNaPi2b_WT	AGTGGATCCATGGCTCCCTGGCCTGA
RNaPi2b_WT	CGGAATTCCTACTCCATCGGCATGAACTCCATCAAGGCCGTGCATTCGGTCT
FNaPi2b_del6	GCC GAA GAA ACT CCA GAA CTG GAT GCG CTC GCT GAA GCC CTG GG
RNaPi2b_del6	CCC AGG GCT TCA GCG AGC GCA TCC AGT TCT GGA GTT TCT TCG GC
FNaPi2b_T330V	CTC CCC TTC CCT CTG TTG GGT GGA TGG CAT CCA AAA CTG GAC
RNaPi2b_T330V	GTC CAG TTT TGG ATG CCA TCC ACC CAA CAG AGG GAA GGG GAG

longs to type II family of sodium-dependent phosphate transporters (SLC34 family). It is involved in regulating homeostasis of inorganic phosphate in human body by intestinal Pi absorption, whereas homologous sodium-dependent phosphate cotransporter NaPi2a is critical for renal Pi reabsorption [10].

NaPi2b is a transmembrane protein with molecular weight in 76–110 kDa range depending on the state of glycosylation [6–9, 11, 12]. It is predicted to be anchored to the plasma membrane through at least 8 highly hydrophobic -helical regions [13]. It has been previously proposed that NaPi2b possesses a large extracellular loop (188–360 aa), 8 transmembrane domains and the N-and C-therminal cytoplasmic tails. The largest extracellular loop contains several potential sites of glycosylation and a region rich in cysteine residues, which might be involved in disulfide bond formation [2].

We have recently described the production of several monoclonal antibodies directed against NaPi2b extracellular loop (188–360 aa) and narrowed down their epitopes between amino residues 311 and 340 [14]. These antibodies might have a therapeutic value, since NaPi2b is a membrane protein and is overexpressed in ovarian cancer.

The recent studies provided the evidence that mutations in *SLC34A2* gene are associated with pulmonary alveolar microlithiasis (PAM) which is characterized by the deposition of calcium phosphate microliths in lungs [15]. To date, there are no data which could link mutations in *SLC34A2* gene to malignant transformation.

In this study we describe two mutations in NaPi2b gene that could be potentially associated with ovarian cancer: T330V in a large extracellular loop and a 6 aa deletion in the C-terminal end of transporter. These mutations, as well as others were identified by bioinformatic analysis of NaPi2b sequences in various DNA data bases. Furthermore, we have created expression constructs of wild type and mutant forms of NaPi2b suitable for making stable cell lines. High level of expression of wild type and mutant forms of NaPi2b in established HEK293 stable cell lines was confirmed by Western-blot analysis. Generated cell lines will be used to study the regulation of NaPi2b under various experimental conditions, such as mitogenic stimulation, treatment of cells with signal transduction inhibitors, exposure to cellular stresses etc.

Material and Methods. *Bioinformatic approaches*. GeneBank data bases were searched for potential mutations in sodium-dependent cotransporter NaPi2b. CLUSTALW (1.82) program (www.ebi.ac.uk/clustalw/) was used for multiple sequence alignment of different EST clones corresponding to NaPi2b.

Cloning of wild type NaPi2b into pcDNA3.1. The full length cDNA clone of human NaPi2b (NaPi2b_WT) was amplified from the original clone DKFZp6860655Q2 (received from RZPD gene bank) with primers containing cloning sites and sequences for the EE-tag (Table). The amplified cDNA fragment was then ligated into mammalian expression vector *pcDNA3.1+* (Invitrogen, USA) that allows the expression of cloned cDNA in mammalian cells under the control of the CMV promoter. Generated cDNA plasmids were confirmed by restriction analysis and DNA sequencing. Plasmid DNA used in subsequent studies was purified by DNA purification kit (Promega, USA).

Site-directed mutagenesis. 20 ng of the *pcDNA3.1*/NaPi2b plasmid was amplified with 2.5 U of Pfu DNA polymerase in the presence of overlapping primers for mutagenesis (9 pmol of each). The primers contained a mutation (ac 988 gt for T330V and del1768–1785 nt for del6aa, see Table) in the middle of the sequence. PCR amplification was performed in 50 l with 18–22 thermal cycles (95 °C for 30 s, 55 °C for 1 min and 68 °C for 16 min). Amplified DNA was precipitated, redissolved in 15 l of water and then the parental *dam*-methylated DNA was digested with 10 U of *Dpn*I for 1 h at 37 °C. 100 l of XL1-Blue ultracompetent cells were transformed with 4 l of the reaction mixture, grown for 45 min in SOC medium and plated onto LB-ampicillin plates. Plasmid DNA was purified by DNA purification kit (Promega, USA). Generated mutations were verified by sequencing analysis.

Production of stable HEK293 cells. Initially, the produced DNA constructs were linearized with *ScaI* restriction enzyme (Fermentas, Lithuania) according to the manufacturer's recommendations. Transfection of HEK293 cells with FuGene (Roche, Switzerland) was performed in 6 cm plates when cell density reached 60–70 %. 5 g of each plasmid DNA (*pcDNA3.1*/NaPi2b-WT, *pcDNA3.1*/NaPi2b-T330V, *pcDNA3.1*/NaPi2b-D6 aa or empty vector) was mixed with 500 l of standard DMEM medium. FuGene reagent (10 l) was added to each sample and incubated at room temperature for 10 min before the addition to cells. After 24 h incubation, the medium was replaced with complete DMEM medium (10 % FBS, 1 mM Glutamine, penicillin (50 U/ml)/streptomycin (0,25 g/ml) antibiotics). After 48 h, the medium was replaced with complete DMEM medium containing 1mg/ml G418 antibiotic (Gibco, USA).

Transfected cells were cultured in the presence of G418 for 7–10 days in order to eliminate nontransfected cells. The generated stable cell lines were cultured in the presence of G418.

Cell lysis and Western-blot analysis. Stably transfected HEK293 cells were lysed in buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM $MgCl_2$, 0,5 % NP-40, and a mixture of Halt Protease Inhibitor Cocktail (Pierce, USA). Protein concentration was measured by Bradford assay (Pierce, USA), and equal amounts of proteins (10 g) were separated in 8 % SDS-PAGE and blotted to polyvinylidene difluoride (PVDF) membrane (Millipore, USA). The membrane was blocked with 3 % BSA in PBS (phosphate-saline buffer) containing 0,1 % Tween-20 (PBST) for 1 h. Anti-NaPi2b and anti-EE-tag antibodies were incubated with membranes at 4 °C overnight. Generation of monoclonal antibodies against the extracellular loop of transporter was previously described [5]. After washing with PBST, HRP-conjugated goat anti-mouse lgG 1:5000 (Promega, USA) was added to the membrane for 1 h at RT. Western blots were developed using the ECL system (Amersham, Sweden) and then exposed to Agfa X-ray film.

Results and Discussion. We have recently identified sodium-dependent phosphate cotransporter NaPi2b as MX35 antigen which is overexpressed in 90 % cases of human epithelial ovarian cancer [2, 3]. In normal cells, NaPi2b mediates the trans-epithelial efflux of inorganic phosphate and sodium ions across the apical membrane of entherocytes in small intestine and plays an important role in the maintenance of phosphate homeostasis in human body [16]. NaPi2b is also expressed on the apical surface of epithelial cells in other organs to provide an appropriate inorganic phosphate level in alveolar surfactant [6], bile [7], saliva [9], and epididymal fluid [11]. Notably, NaPi2b is expressed at a very low level in normal ovary, in contrary to the high expression in epithelial ovarian cancer [1, 5]. So far, the rationale for high level expression of NaPi2b transporter in ovarian cancer is not clear. This might reflect the increased demand in cancer cells for inorganic phosphate which is required for biosynthetic processes and signal transduction. The function of phosphate transporter is known to be regulated by diverse extracellular stimuli, including FGF 23, EGF, glucocorticoids, vitamin D and estrogens [17–21]. Therefore, deregulation of signaling pathways induced by oncogenic transformation may lead to the augmentation of nutrients uptake through the increased expression of transporters at the level of transcription and translation.

We have performed detailed bioinformatic analysis of potential mutations in *SLC34A2* gene in available databases and composed the map of sequence variations in human NaPi2b sequence (data not shown). This study allowed us to identify 15 differences in the

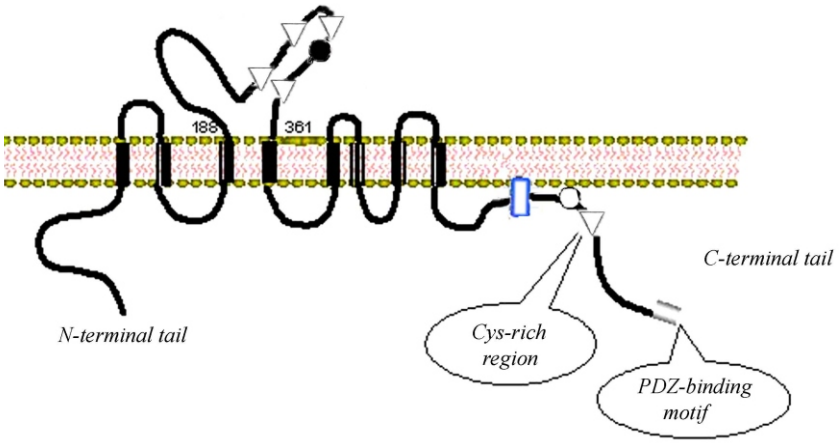

Fig. 1. Schematic representation of NaPi2b domain organization and the location of T330V substitution, 59 and 6 amino acids deletions: – cysteine residue; □ – deletion of 461 ... 519 amino acids (59 amino acids in C-terminus); ● – T330V substitution; ❍ – deletion of 591 ... 596 amino acids (6 amino acids in C-terminus)

coding sequence of human NaPi2b: seven of them were found in genomic DNA of the patients suffering from pulmonary alveolar microlithiasis; one in genomic DNA of a patient with testicular microlithiasis; three in cDNA clones from ovarian cancer cell lines and four from apparently normal tissues.

Bioinformatic analysis of NaPi2b sequences from ovarian cancer cell lines revealed three mutations potentially associated with ovarian cancer: a) single amino acid substitution T330V in a large extracellular loop; b) 6 aa deletion (591–596 aa); and c) 56 aa deletion (461–519 aa) in C-terminus of transporter (Fig. 1, see inset).

A point mutation T330V is located in a large extracellular loop of NaPi2b protein and therefore could influence antigenic properties of transporter. Corut et al. have described T330M substitution in NaPi2b and have indicated that this mutation might inactivate NaPi2b transporter due to the substitution of polar residue to non-polar one [15]. So, this position may represent a hot spot of mutation in NaPi2b, especially in ovarian cancer.

The identified deletions in NaPi2b are located in the C-terminus tail – this region of the phosphate transporter is possibly responsible for the interaction with binding partners implicated in the regulation of cellular localization and function similarly to NaPi2a [22]. A 6 aa deletion is flanked by short direct repeats, which might be involved in the mechanism of mutagenesis by replication slippage [23], site-specific recombination and others. We propose that these mutations may exist in ovarian cancer and may influence NaPi2b cellular localization and function.

We have created mutant cDNA constructs of NaPi2b with a point mutation T330V and a 6aa deletion of 591 … 596 aa by site-directed mutagenesis. Unfortunately, we were not successful in making a 59 aa deletion mutant in mammalian expression plasmid. Cloning of wild type NaPi2b in frame with the N-terminally located EE-tag epitope into *pcDNA3.1* vector was performed as described in Materials and Methods. All generated constructs were linearized and used for stable transfection of HEK293 cells. After 7–10 days selection of transfected cells on geneticin containing medium we have selected colonies for testing NaPi2b expression.

The expression of NaPi2b (wild type and mutant forms) in HEK293 was confirmed by Western-blot analysis of total cell lysates with anti-EE monoclonal antibody (Fig. 2, *A*) or anti-NaPi2b monoclonal antibodies (Fig. 2, *B*). Furthermore, we found that anti-NaPi2b MAb generated against the extracellular loop of transporter L2 (20/3) specifically recognises wild type and a 6 aa deletion mutant, but does not detect the NaPi2b mutant carrying substitution T330V in the extracellular loop of NaPi2b located within a region of epitope for L2 (20/3) MAb (311–340 aa). These data clearly indicate that T330V substitution of hydrophilic to nonpolar amino acid could be crucial for the epitope recognition by L2 (20/3) MAb. It should be noticed that the MX35 epitope is also located in the same region of the large extracellular loop [2] and MX35 MAb does

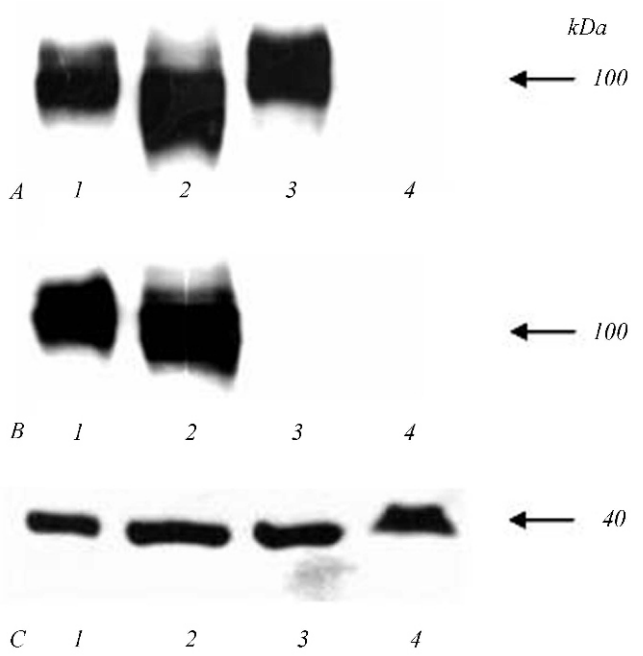

Fig. 2. Expression of wild type and mutant forms of NaPi2b in stably transfected HEK293 cells. WB analysis of HEK293 cells lysates with: *A* – anti-EE-tag antibody; *B* – anti-NaPi2b antibody (L2(20/3); *C* – anti-GAPDH antibody. HEK293 cells transfected with *pcDNA3.1*/NaPi2b-WT (*1*); *pcDNA3.1*/NaPi2b-D6 aa (*2*); *pcDNA3.1*/NaPi2b-T330V (*3*) and *pcDNA3.1* (*4*)

not detect NaPi2b mutant carrying substitution T330V as well (data not shown).

Conclusions. We have created stable cell lines expressing wild-type and mutant forms of NaPi2b phosphate transporter and have shown that T330V mutation in the extracellular loop is not recognized by anti-NaPi2b L2 (20/3) MAb by Western-blot analysis that could be explained by the destruction of the epitope for these antibodies.

The generated stable cell lines will be used for the further analysis of phosphate transporter NaPi2b in normal and transformed cells. We are planning to investigate the impact of generated mutations on the phosphate transport function and cellular processes, such as DNA biosynthesis, growth and proliferation.

The generated stable cell lines will be available for researchers elucidating the function of NaPi2b transporter and those who are studying the inorganic phosphate homeostasis under normal and pathological conditions.

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В. С. Гришкова, Д. С. Літуєв, В. В. Філоненко, Р. Г. Кіямова

Створення клітинних моделей для аналізу Na-залежного фосфатного транспортера NaPi2b – потенційного маркера раку яєчників

Резюме

Мета роботи полягала у створенні клітинної моделі для функціонального аналізу Na-залежного фосфатного транспортера NaPi2b, нещодавно ідентифікованого як маркер раку яєчників. Отримано стабільні клітинні лінії HEK293, які експресують дикий тип, та мутантні форми NaPi2b (точкова заміна T330V у великому позаклітинному домені та делеція шести амінокислотних залишків на С-кінці транспортера), виявлені в клітинних лініях раку яєчника. Експресію дикого типу та мутантних форм NaPi2b у стабільних клітинних лініях підтверджено Вестерн-блот-аналізом за допомогою моноклональних антитіл проти NaPi2b. Описані клітинні моделі можна використовувати для вивчення функцій транспортера NaPi2b за норми та патології.

Ключові слова: Na-залежний фосфатний транспортер NaPi2b, мутація, моноклональні антитіла проти NaPi2b.

В. С. Гришкова, Д. С. Литуев, В. В. Филоненко, Р. Г. Киямова

Создание клеточных моделей для анализа Na-зависимого фосфатного транспортера NaPi2b – потенциального маркера рака яичников

Резюме

Цель работы состояла в создании модели для функционального анализа Na-зависимого фосфатного транспортера NaPi2b, недавно идентифицированного как маркер рака яичников. Получены стабильные клеточные линии HEK293, экспрессирующие дикий тип, и мутантные формы NaPi2b (точечная замена T330V в большом внеклеточном домене и делеция шести аминокислотных остатков на С-конце транспортера), выявленные в клеточных линиях рака яичника. Експрессия дикого типа и мутантных форм NaPi2b в стабильных клеточных линиях подтверждена Вестерн-блот-анализом с помощью моноклональных анти-NaPi2b антител. Описанные клеточные модели можна использовать для изучения функций транспортера NaPi2b в норме и при патологии.

Ключевые слова: Na-зависимый фосфатный транспортер NaPi2b, мутация, моноклональные анти-NaPi2b антитела.

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