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IMMUNOHISTOCHEMICAL ANALYSIS OF NAPI2B PROTEIN (MX35 ANTIGEN) EXPRESSION AND SUBCELLULAR LOCALIZATION IN HUMAN NORMAL AND CANCER TISSUES

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Aim: **To study the expression profile of the NaPi2b protein and its localization in breast, ovarian and lung cancer cells in relation to normal tissues adjacent to tumor.** ***Methods:*** **Immunohistochemical analysis with monoclonal antibody MX35 was applied for investigation of NaPi2b protein expression in breast, lung and ovarian carcinomas. Intensity of NaPi2b protein expression was calculated with semiquantitative scores.** ***Results:*** **NaPi2b (MX35) protein expression was detected in breast, lung and ovarian cancer cells and adjacent normal tissue. We have shown that in contrast to ovarian tumors in breast and lung tumors NaPi2b expression is down regulated comparing to correspondent normal tissues.** ***Conclusion*****: This study provides the data on the pattern of NaPi2b expression and cellular localization in breast, lung and ovarian cancers, which might be useful for understanding the mechanism of transport and maintenance of inorganic phosphate in cancer and normal cells, as well as for developing novel immunotherapeutic approaches based on MX35 monoclonal antibody.**

Key Words: **NaPi2b, MX35 antigen, monoclonal antibody MX35, ovarian cancer, breast, cancer, lung cancer.**

Cancer biomarkers provide diagnostic and prognostic information on diseases that enable interventions with the appropriate therapeutic agents and early decisions for correction of cancer therapy. Furthermore, cancer biomarkers are used as targets for immunotherapy with corresponding monoclonal antibodies, such as ErbB2/Herceptin. The development of therapeutic monoclonal antibodies (mAbs) directed against membrane ovarian cancer antigen MX35, which is overexpressed in 90% of ovarian cancers, is currently in progress. Pre-clinical and Phase 1 clinical trials with radiolabeled mAbs MX35 provided useful information on pharmacokinetics and tolerated dose in patients with ovarian cancer [1, 2]. Recent studies revealed the molecular nature of MX35 antigen as a sodium-dependent phosphate transporter NaPi2b [3]. Sodium-dependent phosphate transporter NaPi2b (*SLC34A2*, NaPiIIb, Npt2) is normally expressed at the brush border membrane of mammalian small intestine and participates in the transcellular inorganic phosphate (Pi) absorption, contributing to the maintenance of phosphate homeostasis in the body [4, 5, 6]. The expression of NaPi2b at the protein level has been detected in the liver [7] and at the apical surface of epithelial cells of mammary, salivary glands [8, 9] and lung [10]. In addition to ovarian cancer [11, 12], the overexpression of MX35/NaPi2b at the mRNA level has been also reported in other human malignancies, including papillary thyroid [13], breast [14] and lung [15] cancers. However, these studies have not been supported by data on NaPi2b protein expression level and its subcellular localization, with the exception of ovarian cancer [16]. There is no doubt that the pattern of NaPi2b expression at the protein level in cancer and adjacent normal tissues may uncover useful information for the application of therapeutic MX35 mAbs.

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MATERIALS AND METHODS

Tissue samples and monoclonal antibody. 63 archive paraffin blocks of serous ovarian cancer (n=10), non-small cell lung carcinomas (n=11) and ductal breast cancer (n=10) as well as cancer adjacent tissues from human breast (n=4), lung (n=9), ovary (n=5), uterus (n=3) and oviduct (n=2) were used for immunohistochemical analysis with mAbs MX35 (Table). MAbs MX35 was kindly provided by Dr. Gerd Ritter from the Ludwig Institute for Cancer Research (New-York, USA).

Immunohistochemical analysis. Immunohistochemistry was performed according to standard protocol. Representative sections of tumor samples were prepared from paraffin blocks and stained with hematoxylin — eosin as previously described [17]. Endogenous peroxidase was quenched with H_2O_2 (3%) in PBS. After blocking of nonspecific binding by avidin–biotin blocking solution (Vector Laboratories, Burlingame, CA, USA), tissue sections were incubated overnight with MX35 mAb (10μg/ml) at 4 °C. Then, sections were probed with biotinylated secondary antibodies for 2h at room temperature (goat anti-mouse biotinylated IgG, Sigma, 1:400), followed by incubation with avidin–biotin–peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The immune complexes were developed with diaminobenzidine solution. Hematoxylin was used for counterstaining. Prepared slides were examined with the use of Zeiss Universal microscope (Zeiss, Germany); images were captured using digital Axiocam software.

Calculations. Positive for NaPi2b (MX35) staining (weak, moderate or strong) in tissues was scored as 1 — single positive cells (<10%); 2 — positive staining in less them one half of cells (10% < 50%); 3 — positive staining in more them one half of cells (>50%) and 0 — as no stained cells at all (Table).

RESULTS

Immunohistochemical analysis of NaPi2b expression and cellular localization was performed with mAbs MX35 on the panel of cancer samples (31) and adjacent normal tissues (23). The intensity of NaPi2b protein expression was calculated by applying a semi-quantitative scoring approach (Table) as indicated above. The definition of NaPi2b (MX35) overexpression was applied when the score of immunoreactive cells was significantly higher in examined cancer samples, when compared to adjacent normal tissues.

Table. Semi-quantitative scoring of immunohistochemical staining for NaPi2b (MX35) in a panel of normal and cancer tissues

Type of tissue	Score			
	0	1	2	3
Normal breast tissue (n = 4)	0	0	1	3
Breast cancer (n = 10)	8	2	0	0
Normal lung tissue (n = 9)	0	0	1	8
Lung cancer (n = 11)	5	5	0	1
Normal uterus tissue (n = 3)	0	0	1	2
Normal oviduct tissue (n = 2)	0	0	0	2
Normal ovary tissue (n = 5)	5	0	0	0
Ovarian cancer (n = 10)	0	1	1	8

Breast. In non-tumor breast tissues NaPi2b was localized exclusively at the apical surface of the gland duct's epithelium (Fig. 1*a*, marked by arrow). Intensity of the staining was predominantly strong and the most of the epithelial cells were stained (Table). Breast cancer tissues were negative in 8 from 10 examined cases (see Table). Cancer cells negative for NaPi2b staining marked by "c" on Fig. 1, *a*, and 1, *b*. NaPi2b positive staining detected in 2 breast cancer samples (Table) was observed on the surface of cancer cells (Fig. 1, c, marked by arrow) or in the nuclei of some cells (Fig. 1, *c*, marked by arrow head). Cells with nuclear localization of NaPi2b sometimes had morphological signs of death (shrinkage or swelling of the cytoplasm and fragmentation of the nucleus). Positive staining of the dead cells was clearly visible in zones of massive necrosis (Fig. 1, *d*, dead cells marked by arrow head).

Lung. Positive staining for NaPi2b expression was found in most epithelial cells (scored as 2 and 3) of normal lung tissues adjacent to cancer cells (Fig. 2, A, and B, marked by "a" and arrows) in all studied cases (see Table). NaPi2b was predominantly localized on the apical surface of the type 2 alveolar cells in normal lung tissue (Fig. 2, *b*, arrow). Lung cancers were NaPi2b negative in 5 samples (Table and Fig. 2, *a*, *c*, 3*a*, cancer cells marked as "c") and positive in other 6 cases.

In the most positive cases of lung cancer NaPi2b staining was observed only in single cells and was scored as 1 (see Table), however in one case weak NaPi2b staining in more than one half of cancer cells was observed (Fig. 3, *b*, and *c*). Despite the fact that membrane localization of NaPi2b in cancer cells

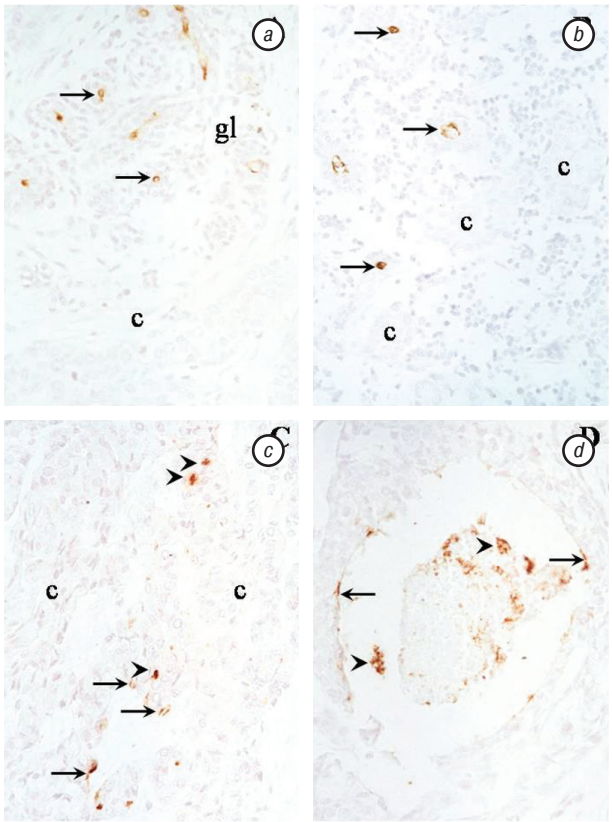

Fig. 1. IHC staining for NaPi2b (MX35) in human breast cancer tissues. *a*, *b*, *c*, *d* — surface staining marked by arrow; *c* — nuclear staining marked by arrow head; *d* — dead cells marked by arrow head; gl — tissue adjacent to tumor; *c* — tumor tissue. Magn. *a-d* — x400

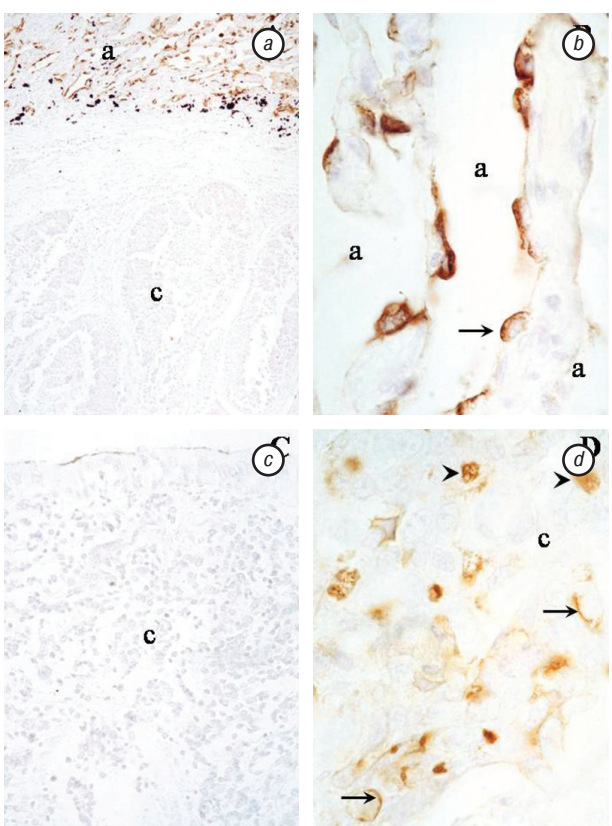

Fig. 2. IHC staining for NaPi2b (MX35) in human lung cancer tissues. *b* and *d* — surface staining marked by arrow; *d* — nuclear staining marked by arrow head; *a* — tissue adjacent to tumor; *c* — tumor tissue Magn. a-x100, *b* and *d* — x1000, *c* — x200

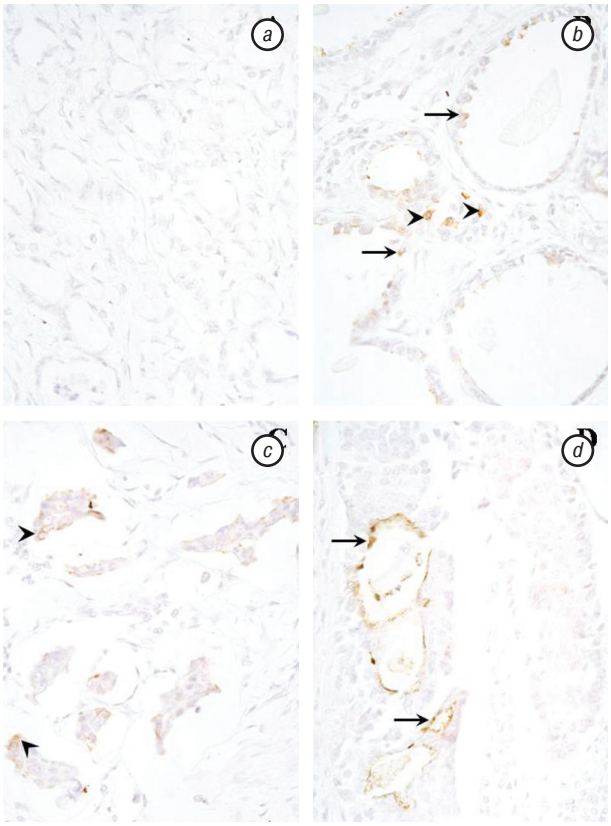

Fig. 3. IHC staining for NaPi2b (MX35) in human lung cancer tissue. *b* and *d* — surface staining marked by arrow; *b* — nuclear staining marked by arrow head, *c* — cytoplasmic staining marked by arrow head. Magn. *a*-*d* — x400

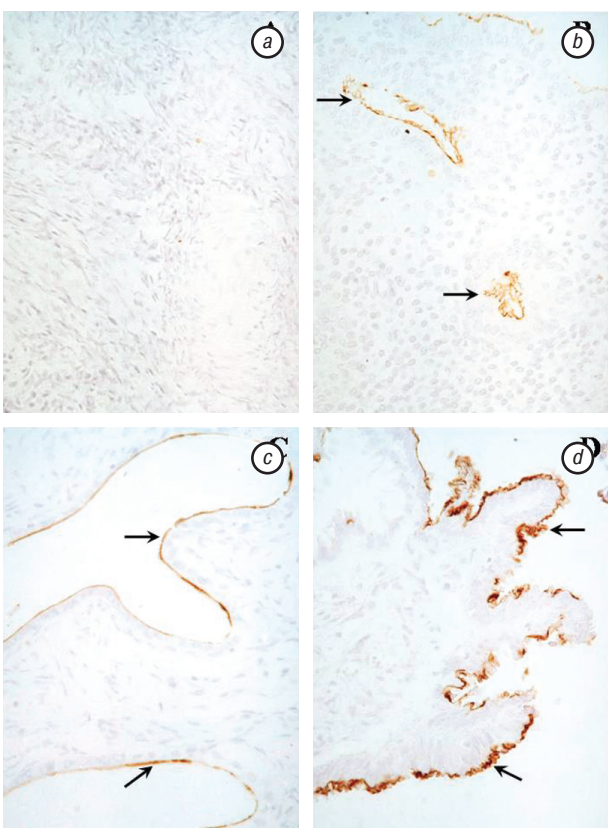

Fig. 4. IHC staining for NaPi2b (MX35) in human normal and ovarian cancer tissues: *a* — ovary, *b* — uterus, *c* — oviduct, *d* — ovarian cancer. NaPi2b staining is marked by arrow. Magn. *a*, *b* and *d* — x400, *c* — x200

is a more common phenomenon for all positive lung cancer cases (Fig. 2, *d,* arrow) nuclear localization of NaPi2b in lung cancer cells was also sometimes detected (Fig. 2, *d*, arrow head). In case of high scored lung adenocarcinoma membrane (Fig. 3, *d*, marked by arrow), nuclear (Fig. 3, *b*, marked by arrow head) and cytoplasmic (Fig 3, *c*, marked by arrow head) NaPi2b positive staining was detected. Zones of massive necrosis in lung cancer samples usually contained positive staining for NaPi2b cells (Fig. 3, *d*) resembling zones of necrosis in breast cancer (Fig. 1, *d*). On the other hand, in zones of invasive growth strong positive staining for NaPi2b with membrane (Fig. 3, *b*, arrow) and nuclear localization (Fig. 3, *b*, arrow head) was observed.

***Ovary*.** No positive staining for NaPi2b (MX35) was found in all studied ovaries (Fig. 4, *a*) while surface epithelium of the uterus (Fig. 4, *b*) and oviduct (Fig. 4, *c*) was intensively stained for NaPi2b (MX35) (marked by arrow). The summary of immunoreactive staining is presented in Table. More precise investigation of ovary must be performed to examine the expression of NaPi2b (MX35) in rudiments (epoophoron, paroophoron, mesonephros). The most of serous ovarian cancer samples showed strong NaPi2b staining of apical surface (Fig. 4, *d*, marked by arrow) of the cancer cells (marked as “c”) (see Table).

DISCUSSION

MAbs MX35 was generated from mice immunized with a cocktail of human ovarian carcinoma cells. The reactivity with a panel of frozen human tissues sections was used as hybridoma selection criteria [18]. Clinical studies with Fab fragments of radiolabeled MX35 antibody suggest their therapeutic potential in patients with ovarian cancer [1, 2]. The murine MX35 antibody and its fragments have been currently investigated in preclinical studies [9] and in a phase I clinical trial in patients with ovarian cancer as the carrier of the alpha-particle-emitting astatine-211 [20, 21]. Molecular cloning of the MX35 antigen as a phosphate transporter NaPi2b was carried out in our laboratory in collaboration with the Ludwig Institute for Cancer Research. This study has broaden the prospects for application of MX35 therapeutic antibodies not only for diagnosis and treatment of ovarian cancer, but also other cancers including thyroid, breast and lung cancers where altered expression of NaPi2b has been recently described at the mRNA level [13, 14, 15]. However, the expression and localization of NaPi2b at the protein level so far has not been investigated in these malignancies.

In this study, we reported the analysis of NaPi2b protein expression in the panel of human cancers and adjacent normal tissues by immunohistochemical analysis with mAbs MX35. In agreement with previously published study [8, 22], we detect the expression of NaPi2b protein in normal lung and breast tissues. In lung, NaPi2b expression was shown on the apical surface of type 2 alveolar cells where it might be in-

volved in the re-absorption from alveolar surfactant Pi, which is subsequently used for phospholipid synthesis [22]. In normal breast tissues, an expression of NaPi2b protein was detected in epithelial cells of secreting mammary glands of caprine, where NaPi2b is possibly implicated in delivering P_i into milk during lactation [8]. We have recently reported that NaPi2b is expressed in oviduct and uterus, but not in ovary [12, 16]. To date, the function of NaPi2b in female reproductive organs was not examined in details. One may expect that NaPi2b is possibly involved in maturation of the egg in fallopian tubes in analogy to the role of NaPi2b in the epididimus where it maintains an appropriate level of inorganic phosphate in epididymal fluid which is essential for sperm maturation and male fertility [23].

Here, we demonstrate for the first time differential expression of NaPi2b on protein level in a panel of human tumors (ovarian, lung and breast) and adjacent normal tissues (see Table 1). We found that NaPi2b protein expression in ovarian, lung and breast cancers is highly heterogeneous and the number of NaPi2b positive cells varies significantly: from no immunoreactive staining to about 100% positively stained cells (see Table). The semi-quantitative analysis of NaPi2b expression revealed that breast and lung cancers express NaPi2b at lower level when compared to corresponding normal tissues. These findings are in agreement with data published by Kopantzev et al. [15], who reported that the expression of NaPi2b mRNA in non-small lung cell carcinoma is significantly lower than that in normal lung tissues. However, our data do not correlate with the study by Chen et al. [14] in which the increased NaPi2b mRNA expression in breast cancer was reported. However, this discrepancy may reflect the difference in NaPi2b expression at mRNA and protein levels. So, further studies are necessary to explore the observed differences.

In agreement with bioinformatic predictions and published studies, NaPi2b showed predominantly cell surface localization. However, nuclear and cytoplasmic NaPi2b staining was also observed in breast, ovarian and lung cancers. Since the function of NaPi2b in cancer cells has not been studied so far, we can only suggest that NaPi2b might be involved in transport of inorganic phosphate via cellular membrane and maintaining its homeostasis. On the other hand the role of cytoplasmic and nuclear fractions of NaPi2b in cellular processes remains unclear and should be further investigated. Notably, nuclear and cytoplasmic NaPi2b staining was mainly observed in zones of invasive growth and massive necrosis. We can only speculate that cell death accompanied by the release of inorganic phosphate may stimulate neighboring cells to increase NaPi2b expression for re-absorption of redundant Pi from extracellular fluid. The increased expression of NaPi2b in zones of invasive growth might reflect the requirement of proliferating cancer cells in Pi, as a critical component of cellular metabolic processes.

In summary, this study provides the data on the pattern of NaPi2b expression and its cellular localization in breast, lung and ovarian cancers, which might be useful for understanding the transport and maintenance of inorganic phosphate in cancer and normal cells, as well as for development of novel immunotherapeutic based on MX35 mAbs. Taking into account that along with evident down regulation NaPi2b expression in breast and lung tumors, in some tumor samples significant number of cancer cells were NaPi2b positive. More precise studies of NaPi2b expression and localization in different tumor breast and lung tumor have to be accomplished.

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