ONCOLOGY

Expression of Heparanase-1 in Prostate Gland Tumors

O. S. Kutsenko, A. V. Kovner*, L. A. Mostovich, I. S. Kunin, R. D. Nepomnyashchikh**, T. Yu. Prudnikova, and E. V. Grigor'eva**

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 152, No. 9, pp. 314-317, September, 2011
Original article submitted June 23, 2011.

Expression of heparanase-1 in prostate tumors was evaluated by RT-PCR, immunoblotting, and immunochistochemistry. Malignant transformation was shown to be associated with considerable increase in the expression of heparanase-1 at both mRNA and protein levels, which correlated with the degree of metastasizing and can be used as the marker for diagnostics of the methastatic process.

Key Words: *prostate cancer; metastasizing; heparanase-1*

Prostate gland (PG) cancer one of the most prevalent tumor diseases in men. High mortality is determined by late diagnosis and high metastatic activity of tumor cells, because metastases are present in more than half cases of primary diagnosis of PG carcinoma due to long-term symptom-free course

There are no diagnostic methods for evaluation of metastatic potential of the tumor. The study of human heparanase-1 gene closely related to metastasizing of tumor cells is promising in this respect. Heparanase-1 cleaves surface heparin sulfates responsible for the maintenance of cell-cell contacts, formation of extracellular matrix, cell adhesion and migration [1]. In normal cells and tissues, expression of heparanase-1 is confined to placenta trophoblasts, hemopoietic cells, and keratinocytes, but malignant transformation is accompanied by considerable stimulation of heparanase-1 expression, which correlates with the increase in invasive properties and metastatic activity of tumor cells for all studied types of cancer [2,8]. Immunohistochemical analysis showed that heparanase-1 is expressed in 90% PG tumors [4] and in 79-94% tumors depending on their malignancy [5]. On the basis of these data, heparanase-1 was proposed as a new molecular marker of metastasizing [7].

Inhibitors of heparanase-1 activity in tumor cells are now intensively studied [9]. Phosphomannopentaose sulfate (PI-88), the most promising preparation, undergoes now phase II clinical trials as an antimetastatic drug [3,6]. These studies are aimed at a decrease in activity of heparanase in the tumor tissue and thereby suppression of tumor invasion, angiogenesis, and metastasizing. However, the expression of heparanase-1 gene in tumors should be preliminary studied for evaluation of the necessity of clinical use of antimetastatic preparations with antiheparanase activity and for choosing optimal strategy of treatment.

Here we compared the expression of heparanase-1 in PG tumors measured by different methods (RT-PCR, immunoblotting, and immunohistochemistry) for choosing the most informative method for clinical diagnostics of PG cancer metastasizing.

Institute of Molecular Biology and Biophysics, Siberian Division of Russian Academy of Medical Sciences, Novosibirsk; *Research Center of Clinical and Experimental Medicine, Siberian Division of the Russian Academy of Medical Sciences; **Research Institute of Regional Pathology and Pathomorphology, Siberian Division of Russian Academy of Medical Sciences, Novosibirsk, Russia. ***Address for correspondence:*** olesyakutsenko@mail.ru. O. S. Kutsenko

0007-4888/12/1523-0344

MATERIALS AND METHODS

Expression of heparanase-1 in clinical samples was measured by RT-PCR, immunoblotting, and immunochistochemical visualization. Specimens of the tumor and visually unchanged PG tissue from 20 patients at the age of 49-68 years with benign hyperplasia (n=7), adenocarcinoma (n=7), and adenocarcinoma with metastases (n=6) were used. All patients signed informed consent for participation in the study. The study was performed in strict compliance with the moral, ethical, and scientific principles governing clinical research as set out in the Declaration of Helsinki (1989).

For RT-PCR analysis of heparanase-1 mRNA level, total RNA was isolated from 50-100 mg tissue using TRIZOL reagent (Invitrogen) according to manufacturer's instructions. Reverse transcription was carried out with 1 μg RNA, oligo(dT)-primers, and M-MLV reverse transcriptase (Promega WI) as specified by the manufacturer. Expression of heparanase-1 gene was analyzed using multiplex PCR on a Tertsik amplifier (DNK Tekhnologii) over 10 cycles, after that primers for *GAPDH* were added and 22 cycles were additionally performed. For amplification, initial denaturation for 5 min at 95°C was followed by 32 cycles consisting of denaturation for 30 sec at 95°C, primer annealing for 30 sec at 59°C, elongation for 60 sec at 72°C, 10 min at 72°C.

The following primers were used: GAPDH-F – 5'-GGGCGCCTGGTCACCAG-3' and GAPDH-R – 5'-AACATGGGGGCATCAGCAGAG-3' (350 b.p. PCR product); HPSE1-F – 5'-GTAGTGATGCCATGTAACTGAATC-3' and HPSE1-R – 5'-TTCGATCCCAAGAAGGAATCAAC-3' (585 b.p. PCR product). PCR products were analyzed by horizontal gel electrophoresis in 1.8% agarose gel in TBE buffer (89 mM tris HCl, 2 mM EDTA, 89 mM boric acid, pH 8.0) containing 1 μg/ml ethidium bromide. DNA marker (1 kb, Fermentas) was used as a molecular weight marker. The gels were scanned in UV light

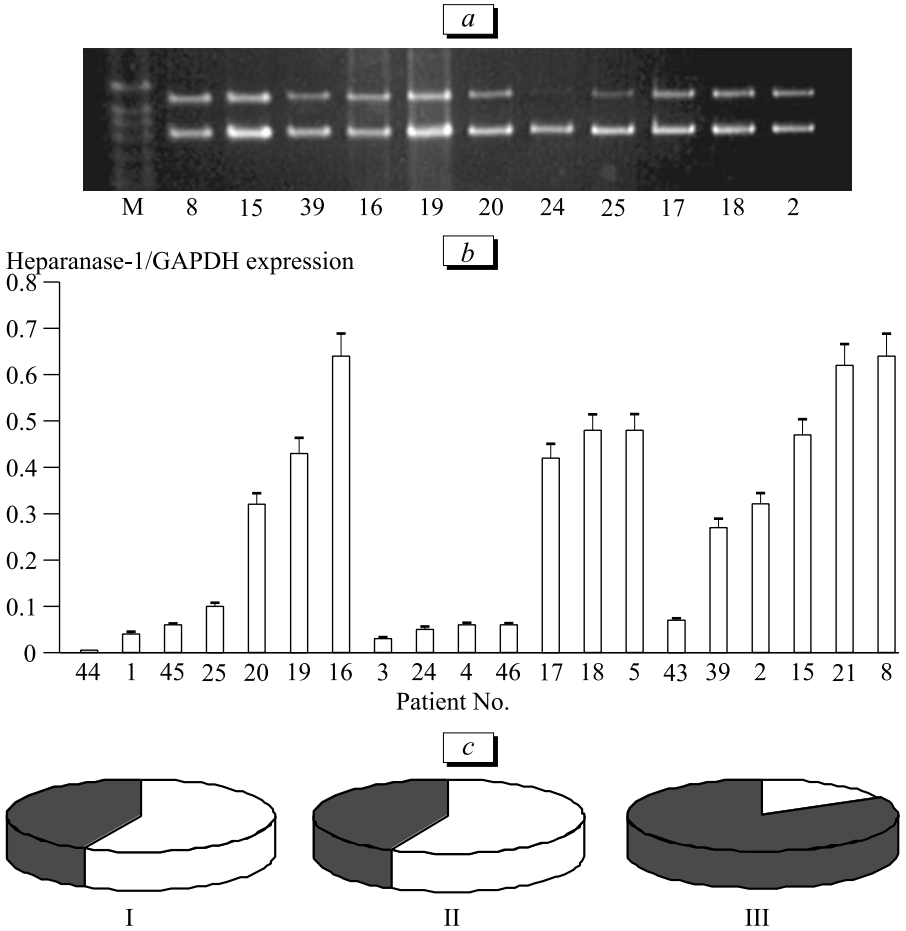

Fig. 1. Analysis of heparanase-1 expression in clinical samples of human PG by RT-PCR. *a*) electrophoresis of products of multuplex RT-PCT for *GAPDH* gene and heparanase-1 gene. 2-39: patients. *b*) expression of heparanase-1 gene relative to *GAPDH* gene expression. 1-45: patients (Nos. 1, 16, 19, 20, 25, 44, 45 – hyperplasia, Nos. 3, 4, 5, 17, 18, 24, 46 – adenocarcinoma, Nos. 2, 8, 15, 39, 21, 43 – metastasizing cancer). Each reaction was repeated 3 times, the data are presented as mean±SD. *c*) diagrams of heparanase-1 gene expression in hyperplasia (I), non-metastasizing (II), and metastasizing adenocarcinoma (III). Light segments: no expression; shaded segments: enhanced expression.

using a DNA Analyzer video system, semiquantitative analysis of gene expression was performed using Total Lab software (Nonlinear Dynamics). The expression of heparanase-1 gene was evaluated in relative units as intensity ratio of amplified heparanase-1 fragment to *GAPDH* fragment.

For immunoblotting, tissue samples were lysed in RIPA buffer (1% Nonidet P-40, 150 mM NaCl, 0.1% SDS, and 50 mM tris, Complete Protease Inhibitor Cocktail tablet (Roche), pH 7.4) on ice. The lysates were centrifuged at 12,000*g* (4°C, 5 min), protein concentration in the supernatant was measured using Quuant-iT Protein Assay Kit (Invitrogen). The protein (35 μg) was applied onto 10% PAAG, separated by electrophoresis (Mini-Protean II Cell-system, Bio-Rad), and transferred to PVDF membrane (Bio-Rad) at 100 V for 2 h. Nonspecific binding was blocked in 5% fat-free milk in PBST at room temperature for 1 h. The membranes were incubated with primary antibodies to heparanase-1 (rabbit anti-human HPSE1, 1:1000, Abnova) for 1 h and then with second antibodies (goat anti-mouse-HRP, 1:2000, Abcam) for 1 h on an orbital shaker. Protein bands were visualized using Opti-4CN Substrate Kit (Bio-Rad) according to manufacturer's instructions.

For immunohistochemical analysis of heparanase-1 expression in the studied samples, paraffin sections were used. After deparaffination, the slides were placed in a humid box and treated with inhibitor of endogenous peroxidase activity (3% H_2O_2 for 30 min at room temperature.). The antigen was demasked with a demasking solution for 30 min at 99°C (Unmasking solution, Vector) followed by blockade of nonspecific binding by incubation in a blocking buffer (PBS×1, 5% BFS) for 30 min at 37°C. Then the slides were incubated with primary antibodies (rabbit anti-human HPSE1, 1:200, Abnova or mouse anti-human GAPDH, 1:250, Invitrogen) for 30 min at room temperature. Visualization of the reaction products was performed using ImmunoPure Ultra-Sensitive ABC Standard Peroxidase Staining Kit (Pierce) system: washout in PBST buffer (3×15 min), incubation with secondary antibodies (anti-rabbit HRP, 1:10,000, Pierce, or anti-mouse HRP, 1:2000, Pierce), and treatment with diaminobenzidine (DAB, 5-15 min at room temperature). Ready preparations were poststained with hematoxylin and eosin and analyzed under an Axiostar Plus microscope (Carl Zeiss).

RESULTS

First we studied the expression of heparanase-1 gene in PG samples from patients with cancer and hyperplasia by multiplex RT-PCR (Fig. 1).

We found that heparanase-1 is expressed in 83% specimens of metastasizing PG cancer and in 43% specimens of PG hyperplasia and PG cancer without metastases. Activation of heparanase-1 expression in metastasizing PG cancer agrees with published data [3,6]. According to our clinical observations, metastases were diagnosed in patients Nos. 2, 8, 15, and 39 and coincided with high expression of heparanase-1 gene in these tumors. This may attest to a possible trend to metastasizing in patients with PG cancer without clinical signs of metastasizing, but with high expression of heparanase-1 in tumor cells (Nos. 18, 17, 5).

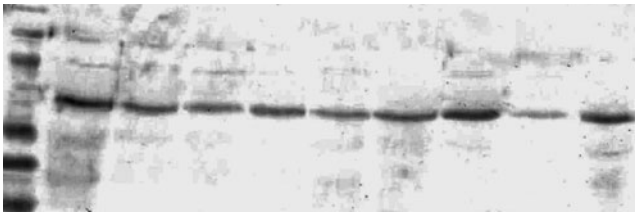

Fig. 2. Immunoblotting of protein molecule of heparanase-1 in clinical specimens of PG: Nos. 1, 25, 45 – hyperplasia, Nos. 3, 5, 18, 24 – adenocarcinoma, Nos. 2 and 39 – metastasizing cancer.

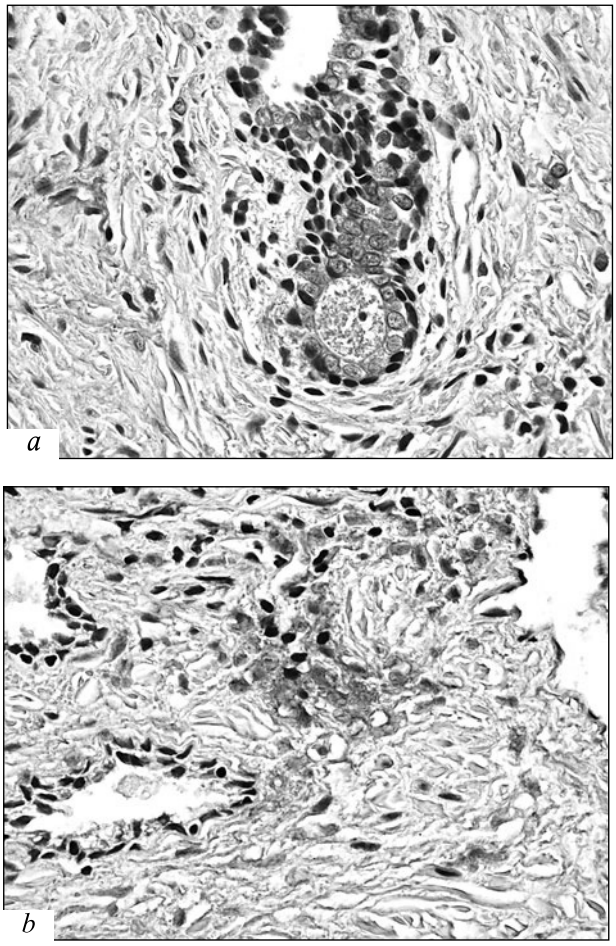

Fig. 3. Immunohistochemical analysis of heparanase-1 expression in specimens of PG adenocarcinoma: *a*) heparanase-1 expression in sample 4, ×630; *b*) heparanase-1 expression in sample 5, ×400.

In further experiments we evaluated heparanase-1 expression in these samples at the level of the protein molecule; to this end, immunoblotting and immunohistochemical analysis were used.

According to our results, immunoblotting is sensitive enough for evaluation of heparanase-1 expression in clinical samples (Fig. 2). We found that expression of heparanase-1 protein molecule varies in different samples, it positively correlated with the level of heparanase-1 mRNA detected by RT-PCR.

Immunohistochemical analysis of paraffin sections prepared from the same specimens of PG cancer and hyperplasia is an alternative method of detection of the heparanase-1 protein molecule (Fig. 3).

We analyzed metastasizing and non-metastasizing PG tumors. It was shown that immunohistochemical detection allows verification of heparanase-1 in the examined tissue and the results well correlate with the results of RT-PCR detection of heparanase-1 mRNA. It should be noted that immunohistochemical analysis showed that presence of heparanase-1 protein not only in the tumor tissue, but also in the adjacent stroma (Fig. 3); moreover, in some samples the positive reaction was detected only in the stroma, but not in tumor cells. These findings somewhat differ from the previous report on the expression of heparanase-1 only in tumor cells [4]. It cannot be excluded that activation of heparanase-1 gene expression in various structures of the tumor tissue can be underlain by different molecular mechanisms and can serve as an additional marker for differential classification of PG tumors.

These findings suggest that immunohistochemical analysis of paraffin sections is the most reliable method for detection of heparanase-1 expression; it not only detects gene expression activation in the tumor, but also allows localization of the cell structures responsible for *de novo* synthesis of heparanase-1 protein molecule.

Thus, heparanase-1 gene is a promising molecular marker of metastasizing and can be used for diagnosis of PG tumor metastasizing and choosing optimal treatment strategy.

The study was supported by Government of Novosibirsk City for Young Scientists (contract No. 25-11).

REFERENCES

1. G. Arvatz, I. Shafat, F. Levy-Adam, *et al., Cancer Metastasis Rev.,* **30**, No. 2, 253-268 (2011).
2. N. Ilan, M. Elkin, and I. Vlodavsky, *Int. J. Biochem. Cell Biol*., **38**, No. 12, 2018-2039 (2006).
3. M. Khasraw, N. Pavlakis, S. McCowatt, *et al., Ann. Oncol*., **21**, No. 6, 1302-1307 (2009).
4. M. A. Kosir, C. C. Quinn, K. L. Zukowski, *et al*., *J. Surg. Res.,* **67**, No. 1, 98-105 (1997).
5. I. Lerner, L. Baraz, and E. Edovitsky, *Clin. Cancer Res*., **14**, No. 3, 668-676 (2008).
6. C. J. Liu, P. H. Lee, D. Y. Lin, *et al., J. Hepatol*., **50**, No. 5, 958-968 (2009).
7. T. Nobuhisa, Y. Naomoto, T. Ohkawa, *et al., J. Cancer Res. Clin. Oncol.,* **131**, No. 4, 229-237 (2005).
8. V. Vreys and G. David, *J. Cell. Mol. Med*., **11**, No. 3, 427-452 (2007).
9. Y. Zhang, L. Li, Y. Wang, *et al., Biochem. Biophys. Res. Commun*., **358**, No. 1, 124-129 (2007).