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MOLECULAR BIOLOGY

Comparative Analysis of *SERPINA1* gene Expression in Tumor Cell Lines[1]

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Abstract—The expression of *SERPINA1* gene in prostate (DU145, PC-3 and LNCaP) and liver (HepG2) tumor cell lines was studied. Alpha1-antitrypsin (AAT) level in the whole cell extracts, secretomes, subcellular fractions and *SERPINA1* mRNA level in the corresponding cells were detected. Discordance between expression at these two levels in PC-3 and LNCaP lines was revealed. A new 37 kDa AAT N-terminus truncated isoform was detected in the nuclear extracts of some prostate tumor cell lines. The mechanism of 37 kDa AAT isoform intracellular retention was proposed. Two polyadenylation sites in the 3'-untranslated region of *SERPINA1* transcripts were identified. A *SERPINA1* gene 3'-untranslated region influence on AAT translation has been discussed.

Keywords: alpha1-antitrypsin, *SERPINA1* transcripts, 3'-untranslated region, alternative polyadenylation, microRNAs.

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The main stage of tumor progression—rearrangement of nuclear processes in transformed cells and formation of stroma guidance mechanisms, which facilitate extracellular matrix degradation, invasion and metastasis maintenance [1]. Proteolytic remodeling of the extracellular matrix is considered as a precondition and consequence of invasive tumor cells migration [2]. Pericellular proteolysis takes central part in mesenchymal movement type of neoplastic cells, that have undergone epithelial-mesenchymal transition [3]. As a consequence, protein repertoire changes not only in transformed cells but also in tumor microenvironment and in the whole organism. Tumor cells modulate the microenvironment in their favor, thus increasing the chances of survival, avoiding immune response and decreasing chances of cell death. Alpha1-antitrypsin (AAT) is one of the potential proteome markers of a transformed cell and its microenvironment. Under normal conditions, AAT is predominantly secreted by hepatocytes, but can also be produced by monocytes, neutrophils, macrophages, alveolar epithelial cells [4]. AAT is the main antiproteolytic agent, protecting tissues and organs from serine proteases and maintaining the essential protease-antiprotease balance.

The following AAT functional state changes may occur while tumor progression: antiproteolytic activity decrease [5] and glycosylation type modulation [6]. Some lung, kidney and colon cancer cell lines secrete AAT with increased oligosaccharide branching [7]. It has been shown that AAT with this type of glycosylation can efficiently inhibit natural killer cytotoxicity, which is the main body defense against transformed cells [8].

Increased AAT levels were found in some tumors. AAT level changes are considered as a diagnostic and prognostic marker. Immunohistochemical study has shown that patients with AAT-positive stomach and lung adenocarcinomas have the least favorable survival prognosis [9]. Elevated AAT secretion was found in blood serum of prostate tumor patients [10].

AAT is encoded by *SERPINA1* gene, which is located in the long arm of chromosome 14 in the region with coordinates 94843084-94857029 in UCSC Genome Browser (gene length 13946 bp) on a parallel DNA chain. *SERPINA1* gene contains 4 coding (exons 2-5), one non-coding exon and a 3'-untranslated region (3'-UTR) with the length of 1702 bp. In UCSC Genome Browser 13 *SERPINA1* transcripts have been annotated, one of them is lacking the exon 5. Probably, there are other transcripts, whose detection is a part of the current study.

Molecular mechanisms of AAT synthesis and transport by tumor cells remain largely unknown.

[1] The article was translated by the authors.

MATERIALS AND METHODS

Prostate cultured cell lines DU145, PC-3, LNCaP and total RNA from RWPE-1 immortalized line of normal prostate epithelium cells were kindly provided by colleagues from N.N. Blokhin Cancer Research Center (Moscow, Russia). HepG2 cell line was kindly provided by colleagues from Engelhardt Institute of Molecular Biology (Moscow, Russia). Cells were cultivated on cell culture plates (Greiner Bio-One, Austria) in DMEM/F12 (DU145, PC-3, HepG2) and RPMI1640 (LNCaP) media supplemented with 10% fetal bovine serum and penicillin/streptomycin solution (100U/ml each) (Gibco, USA) at 37°C, 5% CO_2 in humidified atmosphere. Cells were detached by 0.05% trypsin and 0.913 mM EDTA solution (Gibco, USA) and passaged at 1:4–1:6 ratio each 3–4 days.

Oligonucleotide synthesis and DNA fragment sequencing were performed by "Synthol" company (Moscow, Russia).

Total RNA from cells was isolated by TRIzol (Life Technologies-Invitrogen, USA) following the manufacturer's protocol. RNA concentration and integrity was estimated spectrophotometrically ("Nanodrop", Thermo Fisher Scientific, USA) and electrophoretically.

Relative *SERPINA1* gene expression in total RNA samples was determined by real-time PCR. QuantiTect (Quagen, Germany) system was used for reverse transcription reaction from 1 μg total RNA following manufacturer's protocol. Reaction mixtures without reverse transcriptase were used as control of genomic DNA absence in RNA samples.

Primers for real-time PCR. EX2/3 fragment: forward primer 5'-TGTGGATTTGGTCAAGG-3', reverse 5'-CCTTCACGGTGGTCAC-3'; EX4/5: forward primer 5'-GCATCACTAAGGTCTTCA-3', reverse—5'-GCTTCAGTCCCTTTCTC-3'; EX5: forward primer 5'-GCCATACCCATGTCTATC-3', reverse—5'-GAGCGAGAGGCAGTTA-3'; APA1: forward primer 5'-GCCATACCCATGTCTATC-3', reverse—5'- TTGGGTGGGATTCACCAC-3'; APA2: forward primer as for APA1, reverse—5'-CTCAACCCTTCTTTAATGTC-3'; APA3: forward primer 5'- GACCTGAGAGTCTGAAGA-3', reverse—5'-CTTGAACATCATACCAACTC-3'; *GAPDH*: forward primer 5'-CGTCAAGGCTGAGAAC-3', reverse—5'-ACTCCACGACGTACTC-3'. Real-time PCR was performed in CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA) with Maxima SYBR Green qPCR Master Mix (2X) (Thermo Fisher Scientific, USA) in 20 μl reaction mixture volume. Amplification specificity was estimated by melting curve mode, the products were analyzed in 2% agarose gel. Real-time PCR conditions: pre-denaturation at 94°C—10 min; 45 cycles: 94°C—10 sec, 57.6°C—20 sec, 72°C—30 sec; 94°C 10 sec; melting curve mode: gradual heating from 55 to 94°C with a step of 0.5°C/10 sec. All experiments were done in triplicates.

Amplification efficiency was calculated using standard curve slope (α) obtained by 4-fold dilution series of HepG2 reverse transcription reaction mixture by formula: $E = 10^{[-1/\alpha]}$ or $E(\%) = (E - 1) \times 100\%$. Relative expression levels were calculated by Pfaffl's formula with amplification efficiency accounting modification [12] using *GAPDH* as a housekeeping gene. Amplification efficiency varied from 93 to 99% ($R^2 > 0.99$).

Cells were rinsed with DPBS (Gibco, USA) and lysed with RIPA Lysis Buffer System (Santa Cruz, USA) to obtain whole cell extracts. Secretomes were obtained by cultivating cells in serum-free medium for 48 hours. Nuclear and cytoplasmic extracts were prepared using original protocol that allows one to carefully separate nuclei from cytoplasm without using a homogenizer [13]. Secretomes were concentrated in Amicon Ultra-4 Centrifugal Filter Unit (EMD Millipore Corporation, Germany) system. Total protein concentration in extracts and secretomes was determined by BCA protein assay (Thermo Fisher Scientific, USA). The following antibodies were used for immunoblotting assay: rabbit polyclonal antibodies against AAT C-terminal region (LS-C31768, LSBio, USA) and goat polyclonal antibodies against full-length AAT (ab7633, Abcam, USA) in pair with secondary peroxidase conjugated antibodies 170-6515 (Bio-Rad, USA) and ab6741 (Abcam, USA), respectively. Immunoblotting was performed following Abcam protocol. Membranes were scanned in Chemi-Doc XRS+ (Bio-Rad, USA) system. Commercial AAT 178251 (Calbiochem, Germany) was used as a positive control. Precision Plus Protein WesternC standards (Bio-Rad, USA) was used as protein molecular mass marker.

Real-time PCR data was processed in MS Excel (Microsoft, USA). Immunoblotting data was analyzed in ImageLab 5.0 (Bio-Rad, USA). Data are present as mean value ± standard error of mean.

3'-RACE (rapid amplification of cDNA 3'-ends). 1 μg total RNA treated by DNAse I (Thermo Fisher Scientific, USA) was reverse transcribed by SuperScript III Reverse Transcriptase (Life Technologies-Invitrogen, USA) with primer 5'-GTGCCCTTCGTGCGCTTTTTTTTTTTTTTTTNNN-3' in 20 μl reaction mixture volume. PCR was performed using Expand High Fidelity PCR System (Roche, Switzerland) and the following primers: forward (*SERPINA1* gene exon 5) 5'-GCCATACCCATGTCTATC-3' and reverse adaptor primer 5'-GTGCCCTTCGTGCGCT-3'. PCR conditions: pre-denaturation 94°C—2.5 min; 10 cylces: 94°C—15 sec, 62°C—30 sec, 72°C—2 min; 25 cycles: 94°C—15 sec, 60°C—30 sec, 72°C—2 min with 5 sec increment; final extension 72°C—7 min. Amplification products were analyzed in 1.5% agarose gel, extracted by MinElute Gel Extraction Kit (QIAGEN, Germany) and sequenced.

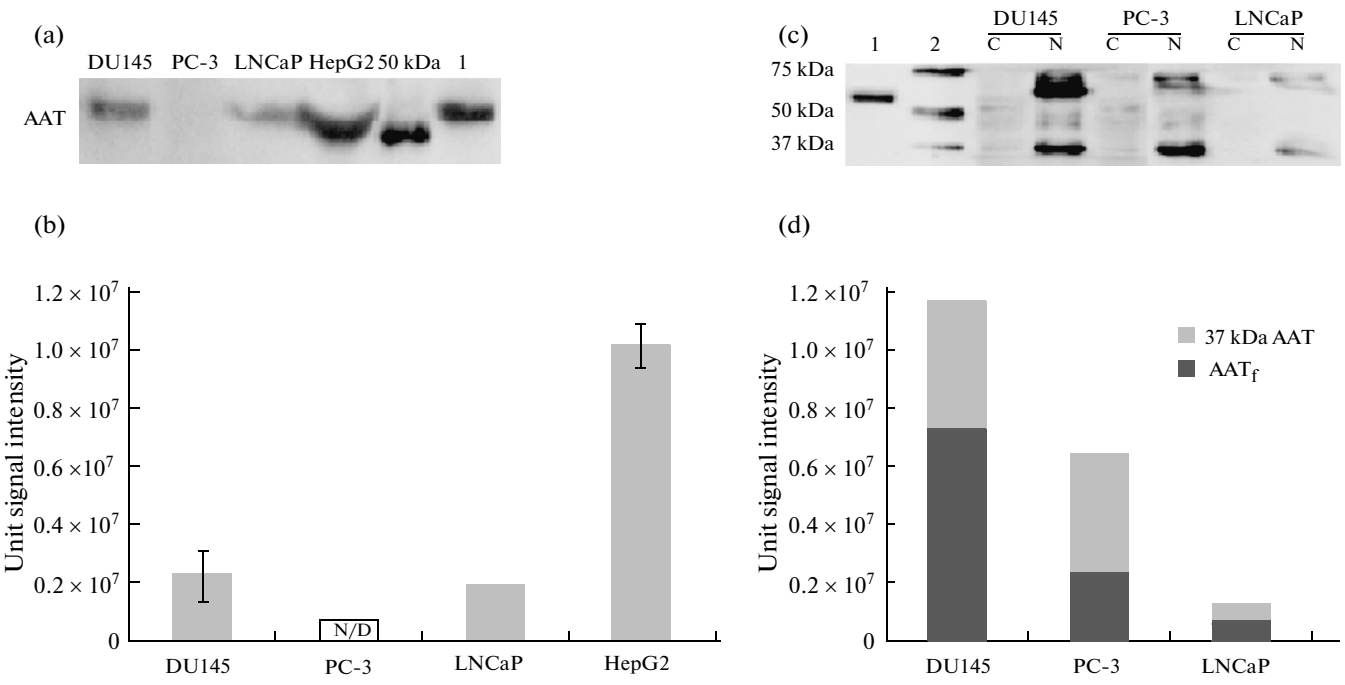

Fig. 1. AAT level estimation. (a) Immunoblotting of cell line secretomes (20 μg total protein per lane): 50 kDa—molecular mass marker, 1—commercial AAT 200 ng. (b) Densitometry analysis diagram of AAT relative levels in secretomes ($n = 3$), N/D—not detected. (c) Immunoblotting of cytoplasmic (C) and nuclear (N) cell extracts (25 μg total protein per lane) using ab7633 antibodies: 1—commercial AAT 100 ng, 2—protein molecular mass marker. (d) Densitometry analysis diagram of nuclear AAT isoform signals: AATf- full-length AAT, signals of AAT > 50 kDa were taken into account.

RESULTS AND DISCUSSION

AAT in whole cell extracts, secretomes (Fig. 1A) and subcellular fractions (Fig. 1c) was detected by immunoblotting. Almost complete absence of AAT in cytoplasm and at the same time its presence in secretomes may be explained by high glucose concentration of 17.5 and 11.1 mM in growing media DMEM/F12 and RPMI1640, respectively. At these concentrations, glucose appears to be a secretion-stimulating factor [14]. More AAT was detected in the nuclear fractions of prostate cancer cell lines than in cytoplasm (Fig. 1c, d), which indicates AAT nuclear translocation. A new 37 kDa AAT isoform was detected in nuclei of the same cells. Binding of antibodies against AAT C-terminus indicates that this isoform is truncated at the N-terminus. It is known that AAT precursor length is 418 amino acid residues with the secretion signal peptide of 24 amino acid residues at the N-terminus. Mature AAT contains 394 amino acid residues and about 15% of its mass is formed by hydrocarbon modifications [14]. Glycosylation of asparagine residues at 46, 87 and 247 positions of AAT polypeptide chain, which are encoded in exons 2 and 3 (Fig. 2a), is necessary for efficient AAT secretion [16]. It was supposed that AAT 37 kDa isoform loses the ability for secretion due to the loss of secretion signal peptide, and this isoform has not been detected in secretomes (data not shown). It was also supposed that 37 kDa isoform may be translated from mRNA truncated at the 5'-end thus not containing some regions of 2 and/or 3 exons. In order to check this hypothesis the relative expression levels of distinct coding regions of *SERPINA1* gene were determined. Exon 2–3 (EX2/3), exon 4–5 (EX4/5) junctions and the region of exon 5 (Fig. 2b) were amplified by real-time PCR. Total RNA from Hep2 cell line was used as a positive control. The secreted AAT relative level, normalized to total secreted protein (Fig. 1b), correlates with real-time PCR results (Fig. 2b). It was found that expression levels of individual coding regions of *SERPINA1* gene in total RNA samples were different (Fig. 2b). These data may indicate the presence of *SERPINA1* transcripts without EX2/3 region, which may correspond to the 37 kDa AAT isoform. The existence of *SERPINA1* transcripts containing only exons 4 and 5 and/or only exon 5 of *SERPINA1* gene also cannot be excluded. It is noteworthy that in immortalized cells of prostate epithelium (RWPE-1 cell line) the levels of all amplified coding regions of *SERPINA1* gene were equivalent (Fig. 2b), so we can suggest that putative truncated transcripts are specific for tumor cells.

In LNCaP cell line EX2/3 and EX4/5 expression levels are higher than in PC-3 cells. Immunoblotting analysis of nuclear and cytoplasmic extracts has shown that the full-length AAT and 37 kDa AAT isoform are higher in PC-3 than in LNCaP (Fig. 1c, d). Such a discordance may be explained by more efficient translation of *SERPINA1* transcripts in PC-3 cells.

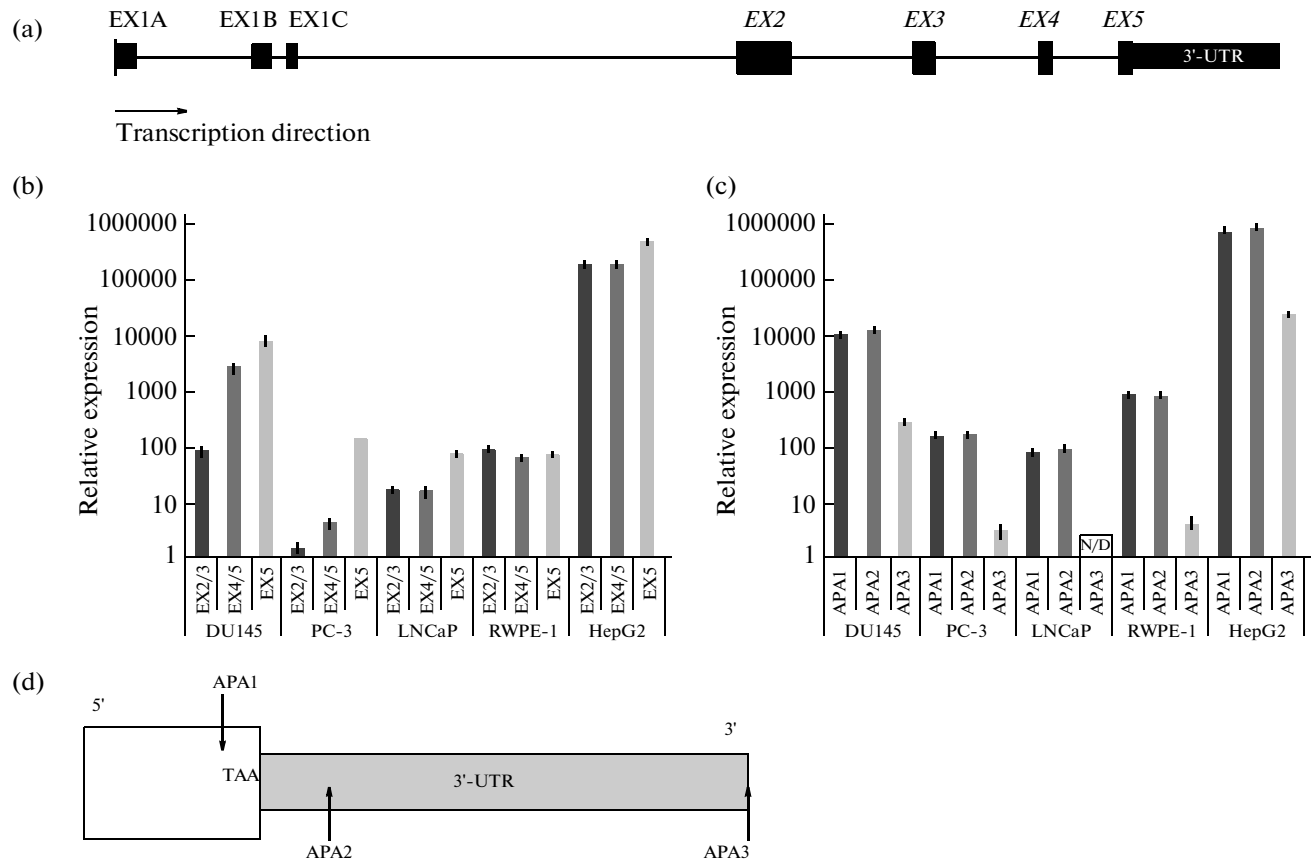

Fig. 2. (a) *SERPINA1* gene scheme: black rectangles—exons, coding exons are marked in italic, untranslated exons—in regular font. *SERPINA1* gene relative expression levels determined by real-time PCR: (b) coding region fragments; (c) *SERPINA1* gene 3'-UTR regions adjacent to alternative polyadenylation sites. Expression levels normalized to *GAPDH* are represented in logarithmic scale. Minimal EX2/3 fragment level in PC-3 cells (b) and APA3 fragment level in RWPE-1 cells (c) were taken as 1; N/D—not detected. (d) Scheme of APA sites' location in *SERPINA1* gene 3'-UTR. White rectangle—exon 5 fragment, TAA stop-codon.

Polyadenylation sites for *SERPINA1* transcripts in total RNA of tumor cell lines were revealed by 3'-RACE and further fragment sequencing. Two alternative polyadenylation (APA) sites were detected (Fig. 2d) in addition to the distal site APA3, found earlier (GenBank Accession numbers JC537036-46). Relative expression levels of the 3'-UTR regions corresponding to three polyadenylation sites were determined by real-time PCR (Fig. 2c). For APA1 and APA2 sites fragments from distal regions (toward the considered) APA2+APA3 and APA3, respectively, are amplified simultaneously. Two proximal sites APA1 and APA2 are mainly involved in polyadenylation for all examined samples. In PC-3 cell line "coding region" level is lower than in LNCaP (Fig. 2b), but APA1 and APA2 region levels are higher, resulting in major part of transcripts with shortened 3'-UTR. It is well known that 3'-UTR shortening predetermines higher transcript stability and translation efficiency [17]. Polyadenylation at APA1 site gives non-stop transcripts, which are unstable and undergo a decay [18], thus APA2 seems to be the main proximal polyadenylation site.

Proximal APA site usage leads to the loss of microRNA binding sites in 3'-UTR, thus avoiding translational repression. Negative regulation of *SERPINA1* gene by miR-940, which has four binding sites in its 3'-UTR, is most likely [19]. Proximal miR-940 binding site is located in transcripts with APA2, others are exclusive for APA3 using transcripts. Distal miR-940 binding site is thought to be the most "active", thus maximal translational repression by miR-940 may occur on *SERPINA1* transcripts with longer 3'-UTR [19].

As previously shown, various physiological states may impact the APA choice: proliferation [20], differentiation and embryogenesis [21] and pathological processes, primarily carcinogenesis [17]. We suppose that 3'-UTR shortening of *SERPINA1* transcripts is one of the changes that happen to cell expression mechanism during carcinogenesis.

Comparative analysis of *SERPINA1* gene expression at mRNA and protein levels allows one to preliminarily estimate the role of AAT in some tumors and

alpha1-antitrypsin potential application as a carcinogenesis marker.

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