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RESEARCH ARTICLE

Mass spectrometry identification of granins and other proteins secreted by neuroblastoma cells

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Abstract We used mass spectrometry-based protein identification to determine the presence of granins and other proteins in the mouse neuroblastoma secretome. We detected polypeptides derived from four members of the granin family: chromogranin A, chromogranin B, secretogranin III, and VGF. Many of them are derived from previously described biologically active regions; however, for VGF and CgB, we detected peptides not related to known bioactivities. Along with granins, we identified 115 other proteins secreted by mouse neuroblastoma cells, belonging to different functional categories. Fifty-six out of 119 detected proteins possess the signal fragments required for translocation into endoplasmic reticulum. Sequences of remaining 63 proteins were analyzed using SecretomeP algorithm to determine probability of nonclassical secretion. Identified proteins are involved in the regulation of cell cycle, proliferation, apoptosis, angiogenesis, proteolysis, and cell adhesion.

Keywords Granins · Neuroblastoma · Secretome · Chromogranin · Secretogranin

Introduction

Over the last few years, there has been a growing interest in the study of cancer secretome comprising all the proteins that can be identified in the intestinal fluid of the tumor mass in vivo which play a key role in the signaling, communication, and migration of cells [24, 33]. The term of "secretome" was introduced by Tjalsma in genome-based studies of *Bacillus subtilis* proteins [51]. Currently, the secretome studies include the proteins secreted via classical and nonclassical pathways but also shed from the surface of living cells [33]. The cell culture secretome can also be a suitable tool for investigating proteins released in vivo by tumors and used to identify putative tumor markers [9]. Neuroblastoma is the most common extracranial solid tumor of the sympathetic nervous system occurring in childhood. This neuroendocrine tumor secretes a range of proteins, which could serve as the potential biomarkers for diagnosis and monitoring of the treatment or disease progression [11, 46]. Several serum prognostic factors, such as neuron specific enolase, ferritin, and chromogranin A (CgA) have been used to predict neuroblastoma progression. CgA is currently the best available biomarker for the diagnosis of neuroendocrine tumors [17, 22, 55]. The granin family comprises nine members including CgA and CgB, secretogranin (Sg) II, III, IV (HISL-19), V (7B2), VI (NESP55), VII (VGF), and proSAAS [15, 16, 18, 56]. Potential utility of CgB, SgII, and VGF nerve growth factor-inducible protein (VGF) as biomarkers of neurological and psychiatric disorders has been described [6]. The expression patterns of granin-derived peptides seem to play an important role in differentiating between some benign and malignant neuroendocrine tumor types [39]. Granins are the main soluble proteins found in many neuroendocrine cells and in some neurons. They are present in large dense-core secretory vesicles and secreted during regulated exocytosis. Granins regulate the storage of catecholamines and ATP, exhibit pH-buffering capacities and thus they help to concentrate soluble products for secretion [7, 18, 32]. Their sequences contain pairs of basic amino acids and monobasic residues that are the potential cleavage sites for proteases. The granin-derived peptides fulfill autocrine and paracrine hormonal activities. Their relative abundance, functional significance, and secretion into the CSF or saliva and the general circulation made granin peptides tractable targets as biomarkers for many diseases of neuronal and endocrine

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origin [6]. We used mass spectrometry-based protein identification to determine the presence of the granin and other protein-derived peptides in the neuroblastoma secretome. This approach could deliver new information regarding neuroblastoma metabolism and new potential biomarkers of the disease.

Material and methods

Sample preparation

The mouse neuroblastoma cell line NEURO-2A was cultured in Eagle's medium with 10 % fetal bovine serum. One-day-old cultures were washed twice with PBS and the serum-free medium was applied. After 24 h culture, media were collected and centrifuged at 3,000×*g* for 30 min. The supernatants were concentrated on centrifugal filters with the molecular weight cutoff of 3 kDa (Millipore, UFC900324). Proteins were precipitated using 5 volumes of cold acetone (−20 °C) and samples were centrifuged at 12,000×*g* for 10 min at 4 °C. Subsequently, pellets were resuspended in 8 M urea and diluted with 25 mM ammonium bicarbonate. Proteins were reduced with 10 mM DTT for 30 min at 57 °C and alkylated with 50 mM iodoacetamide for 45 min at room temperature (RT) in a dark. Then samples were treated with 50 mM DTT for 45 min at RT. Seventy micrograms of protein was used for tryptic digestion and protein identification. Solubilized proteins were digested overnight with sequencing grade modified trypsin (Promega, V5111, 0.01 μg per 1 μg of protein) and the reaction was quenched by adding 0.01 % trifluoroacetic acid.

Mass spectrometry and data analysis

Digested peptides were applied to a RP-18 trapping column (nanoACQUITY UPLC Symmetry C18 Trap, Waters) using 0.1 % trifluoroacetic acid mobile phase, and then transferred on to a HPLC RP-18 column (nanoACQUITY UPLC BEH C18 Column, Waters) using an acetonitrile gradient (0–30 % in 0.1 % formic acid) for 150 min at a flow rate of 200 nL/min. The column outlet was directly coupled to the ion source of the Ion Cyclotron Resonance spectrometer (LTQ61 FTICR, Thermo Electron). For protein identification, a series of three LC/MS runs were carried out on each sample, with the spectrometer running in data-dependent MS-to-MS/MS switch mode. Each run covered one of sectors of *m*/*z* values: 300–600, 500–800, 700–2000.

The parent and product ions lists for the database search were prepared by merging acquired raw files with Mascot Distiller software followed by Mascot Search Engine (Matrix Science, London, UK) against the NCBInr and IPI-Mouse database. Search parameters for precursor and product ions mass tolerance were 30 ppm and 0.8 Da, respectively. The other search parameters were as follows: enzyme specificity was set up to trypsin cleavage and variable modification of cysteine carbamidomethylation and methionine oxidation. Peptides with Mascot score exceeding the threshold value corresponding to <5 % false positive rate, calculated by Mascot procedure, were considered to be positively identified. At least two peptides per protein with score above the threshold were required for identification. The whole experiment was performed twice, using two biological replicates. Functional categorization of proteins was performed using Protein Analysis Through Evolutionary Relationship system (PANTHER, http://www.pantherdb.org/) [36] and Gene Ontology (GO) classification [3]. We determined the presence of the signal peptides and the probability of non classical secretion using the UniProt database [2] and the SecretomeP algorithm [8], respectively.

Electrophoresis and western blotting

Electrophoresis and western blotting was done as it was described previously [29]. Twenty micrograms of proteins per line was used. The monoclonal antibodies for CgA (Chr-A E-5), CgB (Chr-B N-20), SgIII (Sg III C-2), and VGF (H-65) (Santa Cruz Biotechnology, INC) were used according to manufacturer recommendations.

Results

Our mass spectrometry analysis resulted in the identification of four members of the granin family in the pool of proteins secreted by mouse neuroblastoma cells: chromogranin A, chromogranin B, secretogranin III, and VGF nerve growth factor-inducible protein. The peptide sequences and identification parameters are presented in Table 1.

Chromogranin A was identified by detection of seven polypeptides (18 % sequence coverage). Five of them were homologous to the fragments of known biologically active peptides. One peptide (K.ELQDLALQGAK.E) was located within beta-granin/ vasostatin II region; two others (K.TEASEALPSEGK.G and K.DDGQSDSQAVDGD GK.T) were located in pancreastatin region of mouse chromogranin A. Another two polypeptides (K.VAHQ LQALR.R and R.AEDQELESLSAIEAELEK.V) were found in serpinin region. Positions of peptides identified within the protein sequence of chromogranin A are shown in Fig. 1.

We identified eight peptides of chromogranin B with sequence coverage of 16 %. Most of peptides detected for chromogranin B were localized within N-terminal and in the

Table 1 Granins identified in the secretome of mouse neuroblastoma cells

Protein name (NCBI ID)	Protein score	Sequence coverage (%)	Observed mass	Calculated mass	Start–end	Peptide score	Peptide sequence
Chromogranin A	346	18.1	1,034.5865	1,034.5985	453–461	49.74	K.VAHQLQALR.R
gi|6680932			1,044.434	1,044.436	374–382	44.82	R.LEGEDDPDR.S
			1,184.6366	1,184.6401	78–88	36.95	K.ELQDLALQGAK.E
			1,217.5751	1,217.5775	291–302	44.05	K.TEASEALPSEGK.G
			1,334.504	1,334.5109	339–348	51.15	K.QEEEEEEEER.L
			1,492.5831	1,492.5914	276–290	90.83	K.DDGQSDSQAVDGDGK.T
			2,003.0008	2,002.9582	435–452	88.97	R.AEDQELESLSAIEAELEK.V
Chromogranin B	379	16.2	1,114.4636	1,114.4527	399–407	58.32	R.HGEETEEER.S
gi|6680934			1,129.4734	1,129.4822	27–35	36.48	R.DHNEEMVTR.C
			1,197.6101	1,197.6142	438–447	28.67	R.LLDEGHYPVR.E
			1,258.5523	1,258.5499	216–226	42.91	R.ADAHSMELEEK.T
			1,274.5439	1,274.5448	216–226	50.74	R.ADAHSMELEEK.T (Ox. M)
			1,389.6124	1,389.616	300–311	115.4	K.SSYEGHPLSEER.R
			1,468.6466	1,468.6542	386–398	42.24	R.NHPDSELESTANR.H
			1,510.6417	1,510.6059	341–353	48.53	R.ASEEEPEYGEESR.S
			1,530.6815	1,530.6798	102–116	60.17	R.EDAGAPVEDSQGQTK.V
			1,986.9232	1,986.9243	131–148	40.13	R.EGVDDQESLRPSNQQASK.E
Secretogranin III isoform 1	497	23.1	1,473.7428	1,473.7576	233–246	76.77	K.VTPVAAVQDGFTNR.E
gi|6677867			1,539.6937	1,539.7205	441–453	74.49	R.DFINQQADAYVEK.G
			1,765.802	1,765.8118	207–221	40.88	K.EANNYEETLDKPTSR.T
			1,875.9026	1,875.8486	99–115	110.3	R.SPPFDNQLNVEDADSTK.N
			2,734.49	2,734.4388	178–202	143.1	K.LLNLGLITESQAHTLEDEVAEALQK.L
			2,769.4311	2,769.3596	319–343	95.21	K.YGTISPEEGVSYLENLDETIALQTK.N
VGF nerve growth factor	469	13.1	1,009.4654	1,009.4577	423–431	43.85	R.SQEEAPGHR.R
gi|86476054			1,113.5868	1,113.5931	509–519	33.93	R.SPQPPPPAPAR.D
			1,159.548	1,159.5469	353–361	45.38	R.ELQETQQER.E
			1,169.6189	1,169.6193	489–499	36.03	K.NAPPEPVPPPR.A
			1,172.5629	1,172.5686	577–586	40.39	R.HHPDLEAQAR.R
			1,381.7696	1,381.7677	299–311	44.82	R.LLQQGLAQVEAGR.R
			2,034.0237	2,034.0521	466–483	83.11	K.LHLPADDVVSIIEEVEEK.R

middle part of the protein. Two of them (R.DHNEE MVTR.C and R.LLDEGHYPVR.E) were located in $CgB_{1\text{-}41}$ and GAWK regions.

We detected six different peptides of secretogranin III (sequence coverage 18 %). Three of them were related to chromogranin A-binding domain (K.EANNYEETLD KPTSR.T, K.VTPVAAVQDGFTNR.E and K.YGTISPEE GVSYLENLDETIALQTK.N), two were mapped within the cholesterol-binding domain (R.SPPFDNQLN VEDADSTK.N and R.SPPFDNQLNVEDADSTK.N), and one within the carboxypeptidase E (CPE)-binding domain (R.DFINQQADAYVEK.G). For VGF, we detected seven peptides (13 % sequence coverage) including fragments homologous to NERP1-1 (R.LLQQGLAQVEAGR.R) and TLQP-62 (R.HHPDLEAQAR.R). We confirmed the presence of particular granins in neuroblastoma secretome using western blot. Proteins were detected with monoclonal antibodies at the level about 50–70 kDa for secretogranin III, about 70 kDa for chromogranin B and VGF, and 70–80 kDa for chromogranin A (Fig. 2).

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1   MRSTAVLALL LCAGQVFALP VNSPMTKGDT KVMKCVLEVI SDSLSKPSPM
51  PVSPECLETL QGDERILSIL RHQNLLKELQ DLALQGAKER AQQPLKQQQP
101 PKQQQQQQQQ QQQEQQHSSF EDELSEVFEN QSPDAKHRDA AAEVPSRDTM
151 EKRKDSDKGQ QDGFEATTEG PRPQAFPEPN QESPMMGDSE SPGEDTATNT
201 QSPTSLPSQE HVDPQATGDS ERGLSAQQQA RKAKQEEKEE EEEEEAVARE
251 KAGPEEVPTA ASSSHFHAGY KAIQKDDGQS DSQAVDGDGK TEASEALPSE
301 GKGELEHSQQ EEDGEEAMVG TPQGLFPQGG KGRELEHKQE EEEEEEERLS
351 REWEDKRWSR MDQLAKELTA EKRLEGEDDP DRSMKLSFRT RAYGFRDPGP
401 QLRRGWRPSS REDSVEARSD FEEKKEEEGS ANRRAEDQEL ESLSAIEAEL
451 EKVAHQLQAL RRG
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Fig. 1 Localization of identified peptides within chromogranin A sequence

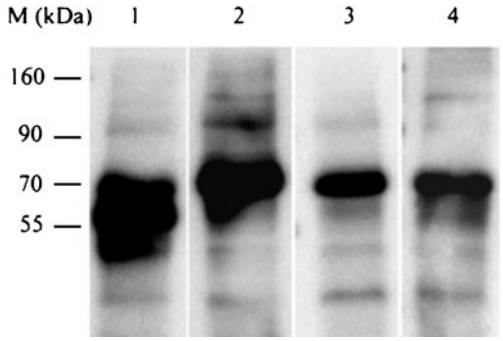

Fig. 2 Western blot detection of granins in the supernatants of neuroblastoma cells. *Line 1* secretogranin III, *line 2* chromogranin A, *line 3* chromogranin B, *line 4* VGF. *M* molecular weight markers

Along with the four proteins belonging to the granins family, we identified 115 other proteins secreted by mouse neuroblastoma cells. Proteins were identified in two runs of identification with at least two peptides detected with the score above the threshold value. Five main molecular function categories of proteins were found by functional categorization using PANTHER system: catalytic (GO:0003824), binding (GO:0005488), structural molecule (GO:0005198), receptor (GO:0004872), and enzyme regulator (GO:0030 234) activities (Fig. 3).

All 119 detected proteins were verified for the presence of the signal fragments in their sequences. Fifty-six of them with signal sequences required for translocation into endoplasmic reticulum are presented in Table 2. The remaining 63 proteins without predicted signal sequence were analyzed using SecretomeP algorithm. We found out 17 proteins with neural network (NN) score above 0.5, which indicates that they could undergo nonclassical secretion (Table 3).

Discussion

In the pool of proteins secreted by mouse neuroblastoma cells, we identified four members of the granin family: CgA, CgB, Sg III, and VGF. The elevated level of CgA was previously found not only in the plasma of patients with neuroblastoma and ganglioneuroma but also with a wide range of tumors like pheochromocytoma; carcinoid tumors of the gastrointestinal tract, lung, and ovary; pancreatic endocrine tumors; and medullary thyroid carcinoma [11]. CgA may play a role in the regulation of tumor angiogenesis, vascular permeability, and endothelial barrier function affecting the response to certain therapies [30]. Human CgA-derived bioactive peptides involve vasostatin I, beta-granin/vasostatin II, prochromacin, chromacin, pancreastatin, catestatin, parastatin, WE-14, and serpinin [1, 12, 19, 28, 34]. We detected five peptides located within bioactive regions of CgA sequence: the peptide K.ELQDLALQGAK.E was located within beta-granin/vasostatin II region, two others (K.TEASEALPSEGK.G and K.DDGQSDSQAVDGDGK.T) were located in pancreastatin region, and another two polypeptides (K.VAHQLQALR.R and R.AEDQELESLSAIEAELEK.V) were found in serpinin region. Increased level of pancreastatin concentrations correlates with tumor differentiation, localized clinical stage, and a favorable outcome for children with neuroblastoma. Kogner et al. suggested that pancreastatin in plasma and tumor tissue can be used as a marker indicating favorable tumor behavior [25]. The newly identified CgA-derived peptide—serpinin—stimulates transcription of protease nexin-1 which is an inhibitor of plasmin protease and its increased expression stabilizes granule proteins in the Golgi complex [26]. Inhibition of plasmin

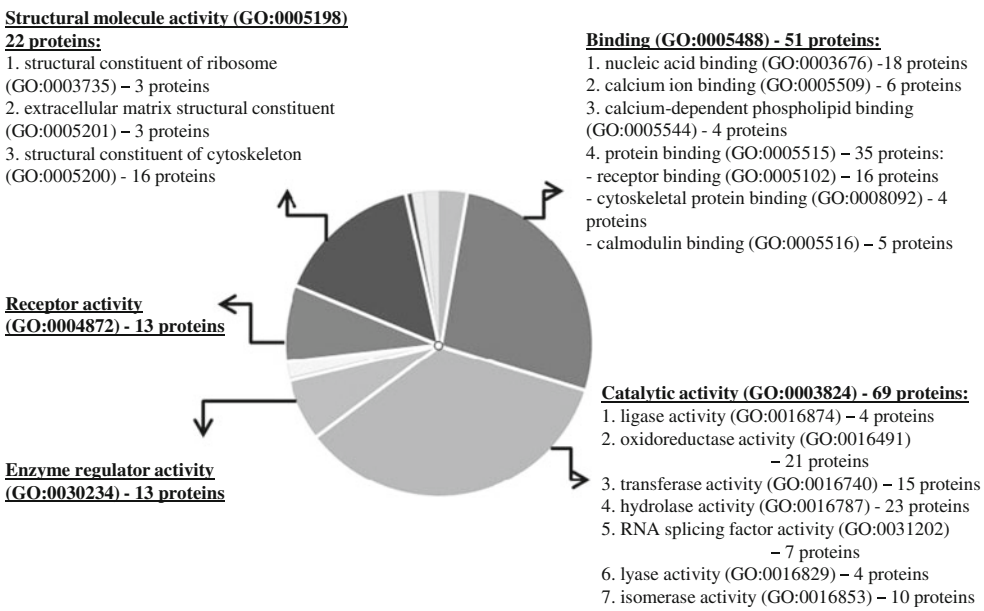

Fig. 3 Functional classification of proteins secreted by mouse neuroblastoma cells using the PANTHER analysis tool (www.pantherdb.org)

Table 2 Proteins with the signal sequence identified in the secretome of neuroblastoma cells

NCBI ID	Protein name	Gene	NCBI ID	Protein name	Gene
gi|1083243	Hypoxia upregulated protein 1	Hyou1	gi|1381582	Sulfated glycoprotein 1	Psap
gi|11066226	Cathepsin Z	Ctsz	gi|13938049	Fibulin 1	Fbln1
gi|11596855	Transferrin receptor protein 1	Tfrc	gi|14250422	Phosphogluconate dehydrogenase	Pgd
gi|12746426	CTF1-alpha	Clstn1	gi|1568625	Laminin subunit alpha-4	Lama4
gi|12841873	Nucleobindin-1	Nucb1	gi|192150	Clusterin alpha chain	Clu
gi|12860234	Lysosomal protective prot.-cathepsin A	Ctsa	gi|2498391	Follistatin-related protein 1	Fstl1
gi|129729	Protein disulfide-isomerase	P4hb	gi|28972103	Peroxidasin homolog	Pxdn
gi|1345609	Bone morphogenetic protein 1	Bmp1	gi|293691	Laminin subunit gamma-1	Lamc1
gi|17390745	Complement C1s-A, light chain	C1sa	gi|38372875	Fibronectin	Fn1
gi|227293	Cathepsin B	Ctsb	gi|396821	Fibulin-1	Fbln1
gi|547841	Low-density lipoprotein receptor	Ldlr	gi|437125	Insulin-like growth factor-binding protein 5	Igfbp5
gi|6678359	Transketolase	Tkt	gi|50409	Chromogranin B (Secretogranin-1)	Chgb
gi|6753556	Cathepsin D	Ctsd	gi|556299	Collagen alpha-2(IV) chain	Col4a2
gi|6755106	Lysyl hydroxylase 1	Plod1	gi|607132	Adipocyte enhancer-binding prot. 1	Aebp1
gi|6755863	Endoplasmin	Hsp90b1	gi|6677867	Secretogranin-3	Scg3
gi|7242187	Legumain	Lgmn	gi|6678077	SPARC	Sparc
gi|9558454	Peptidase inhibitor 16	Pi16	gi|6680932	Chromogranin A precursor	Chga
gi|9790019	Acid ceramidase subunit beta	Asah1	gi|6755144	Galectin-3-binding protein	Lgals3bp
gi|200397	Protein disulfide-isomerase A3	Pdia3	gi|7657027	Dickkopf-related protein 3	Dkk3
gi|309085	Amyloid beta	App	gi|86476054	VGF nerve growth factor inducible	Vgf
gi|1304157	Heat shock 70 kDa protein 5	Hspa5	gi|11762010	Cystatin C precursor	Cst3
gi|114775	Beta-2-microglobulin	B2m	gi|4959705	Fibulin 2	Fbln2
gi|47894398	Tropomyosin 4	Tpm4	gi|125490382	Procollagen C-proteinase enhancer protein	Pcolce
gi|148693781	Neural cell adhesion molecule 1	Ncam1	gi|6753094	Amyloid-like protein 2	Aplp2
gi|53035	Peptidyl-prolyl *cis-trans* isomerase	Ppib	gi|20381317	Aggrecanase-1	Adamts4
gi|6679465	Glucosidase 2 subunit beta	Prkcsh	gi|6175081	Fractalkine	Cx3cl1
gi|10947006	Fetuin-B	Fetub	gi|6680840	Calumenin	Calu
gi|12963609	Sulfhydryl oxidase 1	Qsox1	gi|50852	Granulin-7	Grn

released during inflammatory process may also play a role in protecting cells under adverse pathophysiological conditions. Serpinin and its N-terminally modified form pyroglutamic-serpinin (pGlu-serpinin) also prevent reactive oxygen species and low potassium-induced cell death and hence they may be important in neuroprotection of the central nervous system, neurons, and pituitary cells. Neuroprotective effect of pGlu-serpinin involves activation of signal transduction pathway leading to upregulation of anti-apoptotic Bcl2 proteins [31]. Therefore, serpinins may play an important role in neuroblastoma progression through their anti-apoptotic activities preventing host mediated antitumor mechanisms.

SgIII consists of three functional regions: cholesterol-binding domain, CgA-binding domain, and membrane associated CPE-binding domain [20, 21]. We detected six different peptides of SgIII (sequence coverage 18 %). Three of them were related to CgA-binding domain (K.EANNYEETLDKPTSR.T, K.VTPVAAVQDGFTNR.E and K.YGTISPEEGVSYLENLDETIALQTK.N), two of them were mapped within the cholesterol-binding domain (R.SPPFDNQLNVEDADSTK.N and R.SPPFDNQLN VEDADSTK.N), and one within the CPE-binding domain (R.DFINQQADAYVEK.G). SgIII could mediate between the core aggregate and the cholesterol-rich secretory granule membrane, directing soluble binding cargo proteins to the secretory granules. Proteolytic fragments derived from SgIII have been described, however without specified biological activity [20, 21]. Expression of SgIII in 41 of 47 investigated neuroendocrine tumors was reported [38].

Immunoreactivity of VGF has been found in most well-differentiated neuroendocrinal tumors [39, 41]. Neuroblastoma cells were identified as VGF positive and the VGF expression is upregulated during differentiation [43]. VGF peptides are present in endocrine cells early during development and adulthood and VGF increases in hyperplasia and tumors [41]. Different peptide fragments have been proposed to derive from VGF, including NAPP, NERP,

Table 3 The NN scores rank of identified proteins without the signal sequence (scores calculated using SecretomeP algorithm)

NCBI ID	Protein name	Gene	NN score	NCBI ID	Protein name	Gene	NN score
gi|19111164	Small ubiquitin-related modifier 2	Sumo2	0.883	gi|12844989	Phosphoglycerate mutase 1	Pgam1	0.408
gi|6679108	Nucleophosmin	Npm1	0.803	gi|193442	Galectin-1	Lgals1	0.402
gi|5007032	Transgelin-2	Tagln2	0.790	gi|202423	Phosphoglycerate kinase 1	Pgk1	0.400
gi|226471	Cu/Zn superoxide dismutase	Sod1	0.760	gi|26324898	Eukaryotic translation elongation factor 2	Eef2	0.360
gi|1167510	Ubiquitin	Ubc	0.749	gi|6671539	Fructose-bisphosphate aldolase A	Aldoa	0.357
gi|55291	Vimentin	Vim	0.728	gi|387496	Nucleoside diphosphate kinase A	Nme1	0.349
gi|6755911	Thioredoxin	Txn	0.698	gi|3219774	Peroxiredoxin-6	Prdx6	0.346
gi|42542422	Heat shock cognate 71 kDa protein	Hspa8	0.641	gi|5803225	14-3-3 protein epsilon	Ywhae	0.330
gi|13529464	Nucleolin	Ncl	0.570	gi|45598372	Brain acid soluble prot. 1	Basp1	0.328
gi|6754524	L-lactate dehydrogenase A chain	Ldha	0.568	gi|13569841	Thioredoxin reductase 1, cytoplasmic	Txnrd1	0.324
gi|6755040	Profilin-1	Pfn1	0.560	gi|51452	60 kDa heat shock protein	Hspd1	0.320
gi|6678437	Translationally controlled tumor protein	Tpt1	0.527	gi|4503545	Eukaryotic transl. initiation factor 5A-1	IF5A1	0.319
gi|1517864	Phosphatidylethanolamine binding protein	Pebp1	0.522	gi|6679078	Nucleoside diphosphate kinase B	Nme2	0.307
gi|984938	Proteasome subunit beta type-6	Psmb6	0.512	gi|3914804	Heterogeneous nuclear rnp G	Rbmx	0.300
gi|192050	Aspartate aminotransferase	Got2	0.510	gi|40556608	Heat shock protein 1 beta	Hsp90ab1	0.296
gi|809561	Actin, cytoplasmic 2	Actg1	0.505	gi|3065929	14-3-3 protein gamma	Ywhag	0.290
gi|52865	Lamin-A/C	Lmna	0.505	gi|20178336	Tropomyosin alpha-3	Tpm3	0.261
gi|74178273	Actin, cytoplasmic 1	Actb	0.498	gi|2495342	Heat shock 70 kDa protein 4	Hspa4	0.261
gi|6754910	Nuclear migration protein nudC	Nudc	0.496	gi|6756039	14-3-3 protein theta	Ywhaq	0.256
gi|7106387	Proteasome subunit alpha type-5	Psma5	0.494	gi|115496850	Spectrin alpha 2	SPTA2	0.244
gi|576133	Glutathione *S*-transferase P 1	Gstp1	0.485	gi|1841387	14-3-3 protein zeta/delta	Ywhaz	0.244
gi|202210	Tubulin alpha-1B chain	Tuba1b	0.472	gi|556301	Elongation factor1-alpha1	Eef1a1	0.229
gi|2253159	Peripherin	Prph	0.461	gi|790470	Proliferation-associated protein 2 G4	Pa2g4	0.194
gi|7106439	Tubulin, beta 5	Tubb5	0.458	gi|13384620	Heterogeneous nuclear rnp K	Hnrnpk	0.177
gi|19527048	Heterogeneous nuclear rnp F	Hnrnpf	0.450	gi|6754254	Heat shock protein HSP 90-alpha	Hsp90aa1	0.174
gi|387422	Malate dehydrogenase	Mdh2	0.449	gi|55217	Transitional ER ATPase	Vcp	0.163
gi|115558	Neural cell adhesion molecule L1	L1cam	0.441	gi|14389431	Stress-induced phosphoprotein 1	Stip1	0.155
gi|70794816	Alpha-enolase	Eno1	0.439	gi|2144100	SET nuclear oncogene	Set	0.103
gi|6679439	Peptidyl-prolyl *cis-trans* isomerase A	Ppia	0.421	gi|1711240	Heterogeneous nuclear rnp A1	Hnrnpa1	0.087
gi|1405933	Pyruvate kinase isozymes M1/M2	Pkm2	0.418	gi|3329498	Heterogeneous nuclear rnp A2/B1	Hnrnpa2b1	0.081
gi|1864018	Triosephosphate isomerase	Tpi1	0.418	gi|109866	Nucleosome assembly protein 1-like 1	Nap1l1	0.042
gi|11230802	Alpha-actinin-4	Actn4	0.417				

TLQP, AQEE, and LQEQ [40, 52, 53]. We detected seven peptides of VGF protein, four of them derived from previously described regions: R.LLQQGLAQVEAGR.R (NERP-1), K.NAPPEPVPPPR.A (NAPP-129), R.HHPDLE AQAR.R (TLQP-62/30), and R.SQEEAPGHR.R (APGH). Remaining three polypeptides (R.SPQPPPPAPAR.$D_{(509–519)}$, R.ELQETQQER.$E_{(353–661)}$, and K.LHLPADDVVSIIEE VEEK.$R_{(466–483)}$) are derived from regions without known biological activity. Biological roles of VGF-derived peptides like regulation of energy balance, food intake, body fluid homeostasis, and reproduction were described [4, 5, 13, 23, 45, 52]. Clinical and preclinical data links VGF-derived

peptides in models of human depression (TLQP 62) [49], neuropathic and inflammatory pain (TLQP 21, AQEE 30) [10, 42], amyotrophic lateral sclerosis, Parkinson's, and Alzheimer's diseases [6, 37].

Different CgB peptides were detected in most neuroendocrine tumors [39]. Previously identified peptides derived from CgB include secretolytin$_{(614–626)}$, chrombacin$_{(564–626)}$ and fragment CgB$_{(312–331)}$, CgB$_{(1–41)}$, GAWK$_{(420–493)}$, CCB$_{(597–653)}$, BAM1745$_{(547–560)}$, PE 11$_{(555–565)}$, Sr17$_{(586–602)}$, and Hq34$_{(603–636)}$ [27, 48, 54]. We identified eight peptides of CgB in the pool of proteins secreted by mouse neuroblastoma cells. Most of the peptides detected in our study are localized in N-terminal part of chromogranin B sequence and are not homologous to known bioactive peptides derived from this protein. Two of them (R.DHNEEMVTR.C and R.LLDEGHYPVR.E) were located in regions previously described as CgB$_{(1–41)}$ and GAWK. GAWK-like immunoreactivity is produced by a variety of endocrine tumors and may serve as a plasma tumor marker, especially in patients with pancreatic endocrine tumors [47]. Changes in the level of CgB and derived peptides in CSFs of patients with neurological diseases (e.g., multiple sclerosis, frontotemporal dementia, schizophrenia) were described (for review, [6]). Some conflicting results previously obtained from immunohistological studies using different antibodies may probably indicate differences in processing of particular epitopes in the tumors. That is why using alternative methods like mass spectrometry for investigating of peptides resulted from proteolytic cleavage of proteins ("degradomics") could overcome limitations of antibody-based methods [14].

Along with granins, we identified 115 other proteins secreted by mouse neuroblastoma cells. We examined their sequences for the presence of the extracellular transport signals to determine the mode of secretion. Granins and 52 other proteins with N-terminal signal peptide included in Table 2 can be secreted via the classical pathway (translocation into endoplasmic reticulum, transport through Golgi complex, and secretory vesicles). Remaining 63 proteins could be transported using mechanisms of the nonclassical pathway (import into intracellular vesicles followed by its fusion with the plasma membrane, direct translocation across the plasma membrane, "flip-flop"-mediated secretion of membrane anchored proteins, or secretion in exosomes). To verify the possibility of nonclassical protein secretion, we applied SecretomeP algorithm [8] and we found 16 proteins with NN score above 0.5 (predictions of the non signal peptide triggered secretion, Table 3). However, NN score below 0.5 does not exclude that proteins can be secreted using the nonclassical pathway. So far, only limited number of proteins have been shown experimentally as nonclassical secretory proteins. We detected galectin and thioredoxin which have no signal peptides and were previously described as exported by the alternative pathway [35, 44]; however, NN score for galectin was calculated in the SecretomeP below 0.5.

Functional categorization of detected proteins was performed using PANTHER system (http://www.pantherdb.org/) [36, 50]. Proteins displayed five main molecular function categories: catalytic (GO:0003824), binding (GO:0005488), structural molecule (GO:0005198), receptor (GO:0004872), and enzyme regulator (GO:0030234) activities (Fig. 2). As expected for secreted proteins, a large group was classified as possessing receptor binding or receptor activities and catalytic or enzyme regulatory activities. We also indentified group of proteins with structural molecule activity (GO:0005198) belonging to the structural constituent of cytoskeleton (GO:0005200). The possible contribution of cytoskeletal proteins or their interactions seem to be a potential area for investigating cell communication mechanisms involved in neuroblastoma.

Proteins secreted by mouse neuroblastoma cells can represent different aspects of cancer pathobiology. Among them, we found proteins involved in cell cycle and proliferation (e.g., Nap1l1, Nudc, Vcp, Tubb5, Ywhae, Ywhaq, Ywhag, and Ywhaz), regulation of apoptosis (Clu, Set, Tpt1, Lgals1, Hspa4, and Hspa8), and angiogenesis (L1cam, Pcolce, and Col4A2), proteases, and proteases inhibitors (Ctsa, Ctsb, Ctsd, Ctsz, Cst3, Adamts4, Bmp1, Pcolce, and Lgmn). We found out also a group of proteins originated from cytoskeleton complex (Actg1, Actb, Tpm3, Tpm4, Prph, Vim, Nudc, Tuba1b, and Tubb5) and proteins involved in the regulation of the cell adhesion (Ncam1, Ldlr, Fn1, L1cam, Fbln1, Lama4, Clstn1, and Lamc1). Proteins secreted by cancer cells might play an important role in cancer development and progression. Their analysis can provide insights into the metastasis, angiogenesis, tumor growth, and resistance to antiproliferative signals. Metastatic cascade involves epithelial-to-mesenchymal transition, extracellular matrix degradation, intra- and extravasation, and anoikis evasion. All those steps require specific changes on the level of intra- and extracellular proteins (for review, [24]). Mass spectrometry-based approach to detect and characterize proteins of cancer secretome seems to be a valuable tool in analysis of biomarkers of oncological and neurological disorders. This approach can also supplement antibody-based methods when the protein is affected by differences in epitope processing in cells under pathological conditions. We detected polypeptides from four granins secreted by neuroblastoma cells. Many of them were homologous to previously described biologically active regions of granins; however, for VGF and CgB, we detected peptides not related to known bioactivities. Identification of proteins in extracellular space, involved in cell cycle, proliferation, apoptosis, angiogenesis, proteolysis, and the cell adhesion, may help to explain mechanisms of cell communication during neuroblastoma progression.

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Conflicts of interest None

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