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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

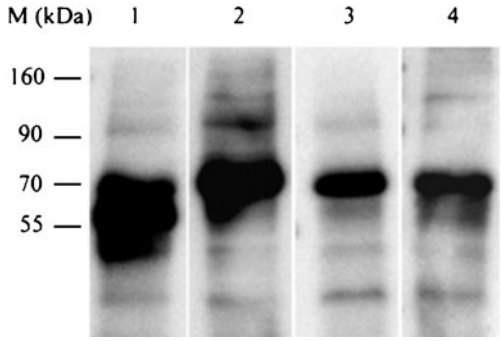


**Fig. 2** Western blot detection of granins in the supernatants of neuroblastoma cells. *Line 1* secretogranin III, *line 2* chromogranin A, *line 3* chromogranin B, *line 4* VGF. *M* molecular weight markers

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

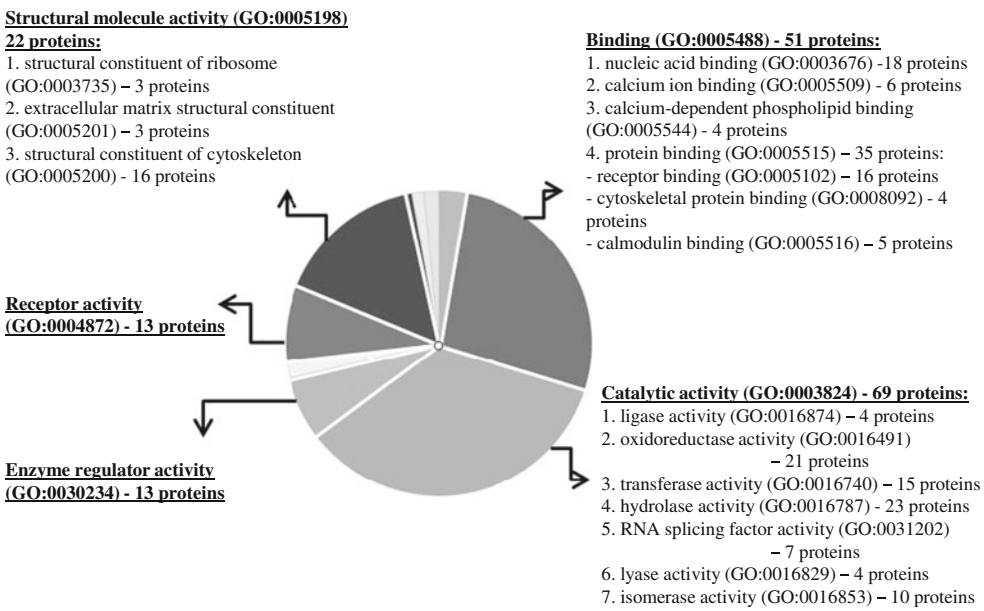


**Fig. 3** Functional classification of proteins secreted by mouse neuroblastoma cells using the PANTHER analysis tool (www.pantherdb.org)

**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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