

Proteome analysis of lipofuscin in human retinal pigment epithelial cells

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Abstract Excessive accumulation of lipofuscin in postmitotic retinal pigment epithelial cells is a common pathogenetic pathway in various blinding retinal diseases including age-related macular degeneration, which is now the most common cause of registerable blindness in the industrialized nations. To better understand the role of lipofuscin accumulation and to manipulate the pathogenetic mechanisms on both experimental and therapeutic levels we analyzed the proteome of isolated human ocular lipofuscin granules from human RPE cells. After homogenization and fractionation by gradient ultracentrifugation of the RPE/choroid complex from 10 pairs of human donors, protein compounds were separated by 2D gel electrophoresis and analyzed using matrix-assisted laser desorption/ionization mass spectrometry and HPLC-coupled electrospray tandem mass spectrometry. Besides a better understanding of downstream pathways, this approach may provide new targets for therapeutic interventions in a currently untreatable disease. © 2002 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Lipofuscin; Proteome; Retinal pigment epithelium; Age-related macular degeneration

1. Introduction

Accumulation of lipofuscin is a common phenomenon in ageing cells [1]. In postmitotic retinal pigment epithelial (RPE) cells, lipofuscin accumulates with age and in association with various blinding retinal diseases including degenerative and hereditary entities [2,3]. Normal RPE cell function is an essential prerequisite for photoreceptor and, thus, neurosensory retinal function [4]. Lipofuscin composition is thought to vary to a large extent in different tissues including neuronal cells of the central nervous system or hepatocytes [1]. In human RPE cells, several lines of evidence indicate that lipofuscin is a potentially deleterious byproduct of the constant phagocytosis of shed distal photoreceptor outer segment (ROS) disks [5]. Normally, degradation of this phagocytic load throughout life occurs in the lysosomal compartment with subsequent release of degraded material at the basal cell side and clearance by the choriocapillaris [5]. The mechanisms of lipofuscinogenesis are currently incompletely understood. The occurrence in a variety of monogenetic retinal

diseases such as Stargardt's disease (mutations in the ABCR gene) and Best's disease (mutations in the VMD2 gene) suggests that excessive accumulation of lipofuscin represents a common downstream pathogenetic pathway of various molecular etiologies [6,7]. There is both experimental and clinical evidence that lipofuscin in this particular cell phenotype is associated with cellular dysfunction and subsequent impairment of visual function in corresponding retinal areas [8,9]. In humans, photoreceptor density was found to correlate with the lipofuscin concentration of the apposing RPE cells [10]. In vivo investigations using confocal scanning laser ophthalmoscopy have demonstrated excessive lipofuscin accumulation in association with various manifestations of age-related macular degeneration (AMD) [11]. The development of atrophy has been found to occur in areas of increased lipofuscin-mediated fundus autofluorescence in a longitudinal study in patients with AMD [11]. Direct evidence that an individual component of lipofuscin interferes with metabolic functions of RPE cells has not been demonstrated until recently [12].

Electron microscopic studies have shown a stepwise conversion of lysosomal structures to lipofuscin granula. Although lysosomal hydrolytic enzymes are capable of degrading most cellular macromolecules, it is assumed that lipofuscin results from an incomplete degradation in secondary lysosomes and/or the presence of altered biomolecules that are not degradable [13]. Lipid oxidation has been suggested to play a role [14], whereby the pigments may arise as a consequence of antioxidant deficiency or under prooxidant conditions [15,16]. Once formed, the RPE cell has no means of degrading or transporting lipofuscin material and granules into the extracellular space via exocytosis. Subsequently, these granules are trapped in the cytoplasm. An age-dependent accumulation of RPE lipofuscin has been described applying various in vitro and in vivo methods [8,17].

Only few compounds of RPE lipofuscin have been identified to date [18]. Current knowledge suggests that lipofuscin is a heterogenous mixture of various biomolecules partially modified by oxidation and lipidperoxidation due to excessive UV-irradiation and high oxygen levels in the eye [19]. More than 10 different fluorophores have been identified [18]. A2-E (*N*-retinylidene-*N*-retinylethanolamine), a major retinoid component of RPE lipofuscin, which represents a Schiff base product of all-trans retinaldehyde and ethanolamine (ratio 2:1) has recently been shown to cause a striking inhibition of major lysosomal catabolic pathways in cultured human RPE cells in concentrations that occur in vivo [12,20,21]. While interference with normal cell function has been elucidated for A2-E, we speculate that lipofuscin granules may contain other potentially toxic compounds. Furthermore, an

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expanded analysis of lipofuscin components may give insights into pathogenetic mechanisms and may, thus, provide new targets for designing effective therapeutic strategies in this currently untreatable blinding retinal disease. Therefore, we sought to determine the proteinous composition of isolated human ocular lipofuscin by applying modern protein analytical methods. To our knowledge this study represents the first description of the human lipofuscin proteome.

2. Materials and methods

2.1. Lipofuscin isolation

Lipofuscin was isolated using a procedure of Boulton et al. [22]. Briefly, the RPE/choroid complex of 10 pairs of eyes derived from donors of corneal transplantations with no known ophthalmologic disease aged between 61 and 97 years were collected and stored at -20°C . Post mortem times varied between 24 and 48 h. The cells of the complex were homogenized on ice in a Potter-Elvehjem-Homogenizer using phosphate-buffered saline (PBSA) and centrifuged at $60\times g$ for 7 min. to remove cellular debris. Then pigment granules were pelleted by centrifugation ($6000\times g/10$ min.), resuspended in 4 ml 0.3 M sucrose, and layered on a discontinuous gradient built of eight different concentrations of sucrose: 2.0, 1.8, 1.6, 1.55, 1.5, 1.4, 1.2 and 1.0 M (4 ml each). Identification of pure lipofuscin in the first band was done by analysis of excitation and emission spectra of isolated lipofuscin as described by Boulton et al. [23] using the high performance spectralfuorometer SFM 25 (Kontron). The first orange-brown band between 1.0 and 1.2 M sucrose containing pure Lipofuscin was collected using a Pasteur pipet. Lipofuscin was resuspended in 0.3 M sucrose and purified on a second sucrose gradient. Dilution in PBSA, centrifugation at $6000\times g$ for 10 min. and washing three times in PBSA followed. The samples were stored at -20°C .

2.2. Protein content of lipofuscin

Lipofuscin-related proteins were extracted from isolated lipofuscin with 0.5 M NaOH. Protein concentration was measured using the method of Lowry et al. applying bovine serum albumin as standard [24].

2.3. 2D gel electrophoresis of lipofuscin

Isoelectric focusing (IEF) was performed using dry polyacrylamide gel strips (IPG, 11 cm) with an immobilized pH gradient pH 3–10 and the IPGphor[®] Isoelectric Focusing System (Amersham Pharmacia Biotech). IPG strips were rehydrated in reswelling buffer [7 M urea, 2 M thiourea, 4% Triton X-100, 65 mM dithiothreitol (DTT), 0.8% Phormalite] overnight. Lipofuscin protein was extracted in reswelling buffer and 80 μl were pipetted into the sample cup of each strip holder. The IEF protocol started at 100 V for 16 h followed by 500 V for 1 h, 500–8000 V (linear increase) within 10 h and finally 8000 V for 4.5 h.

The Multiphor II flatbed system and ExcelGel[®], gradient 8–18 was used for sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) as second dimension. Equilibration buffer for IPG strips contained 50 mM Tris–HCl, pH8.5, 2% SDS, 6 M urea, 30% glycerol and 65 mM DTT for the first 15 min. followed by 260 mM iodoacetamide. After draining the equilibration solution on filter paper the IPG strips and prestained SDS–PAGE standards (Bio-Rad) were placed on the SDS gel. Electrical settings were 600 V, 20 mA, 30 W for 50 min, followed by increase of current up to 50 mA. The gel was finally stained with colloidal Coomassie G50 (Perbio) for 5 days [25].

2.4. Tryptic digestion

Protein spots were excised from the gel and washed twice with deionized water, 50% acetonitrile/water 1:1 and acetonitrile. Proteins were digested with 33–165 ng sequencing grade modified trypsin (Promega) in 40 mM ammonium bicarbonate at 37°C overnight. The reaction was stopped by freezing [25].

2.5. Matrix-assisted laser desorption ionization (MALDI) mass spectrometry

MALDI mass spectra were recorded in the positive ion reflector

mode with delayed extraction on a Reflex II time-of-flight instrument (Bruker-Daltonik GmbH, Bremen, Germany) equipped with a SCOUT multiprobe inlet and a 337 nm nitrogen laser. Ion acceleration voltage was set to 26.5 kV, the reflector voltage was set to 30.0 kV and the first extraction plate was set 20.6 kV [25]. Mass spectra were obtained by averaging 200–300 individual laser shots. Calibration of the spectra was performed internally by a two-point linear fit using autolysis products of trypsin. Sample preparation for MALDI mass fingerprints was achieved by cocrystallization of matrix (α -cyano-4-hydroxy cinnamic acid) with ZipTip C18 (Millipore, Bedford, MA, USA) concentrated samples. Database search was done against the NCBI database using the MASCOT search algorithm (www.matrixscience.com).

2.6. On-line high-performance liquid chromatography (HPLC)-coupled ESI MS/MS

LC separations of the tryptic digests were performed using a Ultimate Capillary/Nano-HPLC system (Dionex, Sunnyvale, CA, USA). After preconcentration using a 0.3 mm \times 1 mm C18 Pepmap column (LC-Packings, San Francisco, CA, USA), the peptide mixture was separated on a 75 μm ID \times 250 mm C18 Pepmap column (LC-Packings) with a flow rate of 190 nl/min and a linear gradient from 8 to 46% acetonitrile in 0.1% aqueous TFA in 55 min. The HPLC system eluent was connected to a Finnigan MAT LCQ ion trap mass spectrometer (ThermoFinnigan, San Jose, CA, USA) equipped with a nanospray source (Protana, Odense, Denmark). Spray voltage was set to 1.5 kV and heated capillary temperature was 200°C . After performing one scan in MS mode from 300 to 2000 Da, the three most intense ions in the scan were selected and subjected to CID. Database search was done using TurboSequest 2.0 (ThermoFinnigan, San Jose, CA, USA).

3. Results

Spectral characteristics of isolated lipofuscin corresponded to previously published spectra of pure RPE lipofuscin (Fig. 1) and light microscopic examination [22,23] of the purified granules confirmed their purity (data not shown). Extraction of lipofuscin isolated from 10 eyes yielded 18.9 mg of protein. 2D gel electrophoresis of extracted proteins resulted in the separation of 81 individual protein spots (Fig. 2). The spots were excised from the gel, digested with trypsin and analyzed by MALDI mass spectrometry. Proteins were subsequently identified by data base search with the obtained peptide mass fingerprints (Table 1). In addition, most of the samples

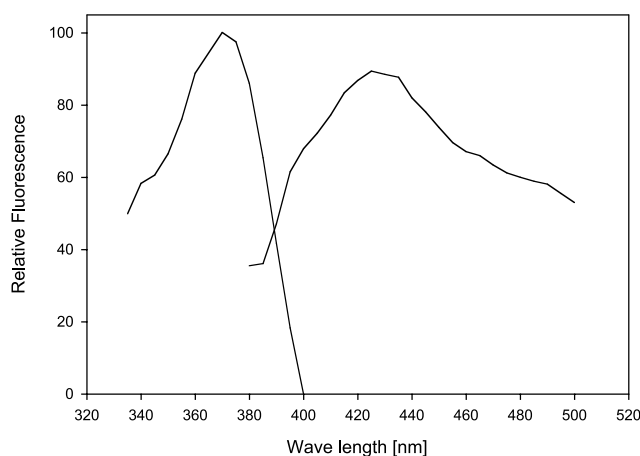


Fig. 1. Excitation and emission spectra of human ocular lipofuscin were recorded on a Kontron SFM 25 fluorimeter. The excitation spectrum was recorded at a constant emission wavelength of 430 nm. For recording of the emission spectrum, excitation was set at 360 nm. Spectra were corrected by subtracting measurements of pure solvent.

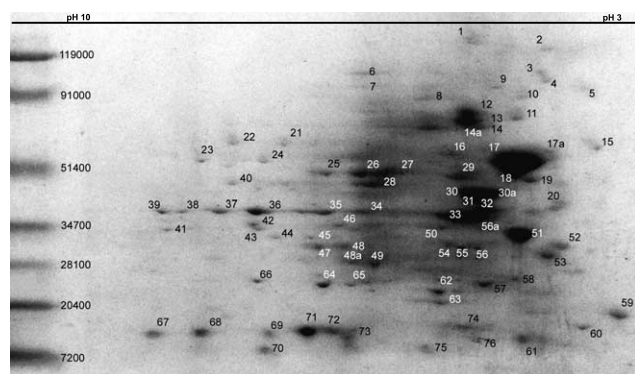


Fig. 2. Separation of lipofuscin proteins by 2D gel electrophoresis in an immobilized pH gradient from 3 to 10 with subsequent colloidal Coomassie staining as described in Section 2. Stained spots were identified by MALDI and ESI mass spectrometry and listed in Table 1 with corresponding numbers. Molecular weight markers (kDa) are shown at the left margin.

were also analyzed by HPLC-coupled ESI MS/MS to confirm the MALDI results by sequence data. Most of the identified proteins represent abundant cellular proteins, including cytoskeleton proteins, proteins of phototransduction, enzymes of metabolism, proteins of the mitochondrial respiratory chain, ion channel proteins and chaperones, which are known to occur in photoreceptors, RPE and other cell types. Remarkable is the occurrence of recoverin (spot 58), since it represents a photoreceptor cell-specific protein [26]. Four spots could not be identified due to missing database hits. Clusters of spots containing the same protein, e.g. spots 12–14, 34–36, 37–39, 47–49, 52–53, 55–56 and 63–65 may represent different post-translationally modified forms. For most of the spots the experimental molecular weight corresponded closely to the theoretical mass as deduced from the database. Only a few spots (20, 29, 31, 54, 57) indicated a lower molecular weight as compared to theoretical data, and one spot (49) exhibited a significantly higher experimental mass. Five spots were identified as hemoglobin chains probably representing blood contaminations during lipofuscin preparation.

4. Discussion

This is, to our knowledge, the first description of the protein components of human ocular lipofuscin isolated from RPE cells. Lipofuscin in postmitotic RPE cells is thought to be mainly a byproduct of the constant phagocytosis and degradation of shedded ROS. Therefore, lipofuscin should contain degradation products of photoreceptor-derived proteins. Indeed most of the identified protein components of lipofuscin have been shown to occur in photoreceptors, but many are also abundant in various other cell types, including RPE. The presence of the ROS-specific protein recoverin [26] underscores the contribution of phagocytosed ROS to lipofuscin. On the other hand, various mitochondria-derived proteins (e.g. in spots 18, 20, 23, 24, 27, 33, 40, 57, 60, 67, 75) were detected in lipofuscin granules, although ultrastructural examinations indicate that mitochondria are concentrated in photoreceptor inner segments, whereas ROS lack these organelles [27]. Thus a considerable proportion of lipofuscin is likely to originate from autophagic processes in the RPE cell itself.

The cytoplasmic space of aged RPE cells or cells with early excessive lipofuscin accumulation may be occupied by lipofuscin granules to large extend. It has been suggested that these vast intracellular deposits in membranous bags may physically impair cellular structure and function [28]. While it has initially been assumed that lipofuscin is merely an inert mixture of various biomolecules, several lines of evidence now indicate that it contains various cytotoxic compounds. Since the majority of the proteins identified herein are cellular house keeping proteins which per se would not be expected to have toxic properties, posttranslational modifications may result in generation of toxic substances. In particular, UV light in association with high oxygen concentrations causing permanent peroxidation of polyunsaturated lipids in the membrane system of photoreceptors finally may result in the generation of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) which are known to modify proteins and initiate crosslinking. Such modification may hinder protein degradation by the lysosomal system, thereby contributing to lipofuscin deposition [13]. Furthermore, deposition of MDA- and 4-HNE-protein-adducts might be actively involved in the pathogenesis of AMD by inducing an inflammatory process [29]. Therefore, such posttranslational modifications of lipofuscin-associated proteins are currently investigated in our laboratory.

For most of the proteins identified, the molecular weight deduced from the position of the spots corresponded to the expected molecular weight. Therefore, intermolecular cross-linking of proteins to form higher complexes is unlikely. For some proteins (spots 20, 29, 31, 54, 57) the position in the gel was shifted to a lower molecular weight, probably indicating the accumulation of proteolytic degradation products in lipofuscin.

Opsin, the major protein component of ROS, was not detected in lipofuscin, suggesting that a high concentration of a protein in the phagocytosed material does not necessarily lead to its accumulation in lipofuscin granules.

The observation that human ocular lipofuscin contains several subunits of ATPase (spots 18, 23, 24, 57), interestingly, shows parallels to Batten disease (juvenile ceroid-lipofuscinosis), an inherited disorder which is characterized by massive lysosomal accumulations of subunit c of the mitochondrial ATP synthase complex. The disease results in total blindness due to retinal degeneration [30]. In an experimental model of the disease, accumulation of subunit c-containing autofluorescent lysosomal storage bodies is linked to severe retinal and neurologic degenerations [31].

Lipids, in particular carotenoids and retinoids, are also characteristic components of RPE lipofuscin and have been implicated in the pathogenesis of AMD. At present, the contribution of single compounds of lipofuscin to disease processes cannot yet be verified. A concerted action of various lipofuscin constituents appears likely. Our present study provides a starting point for further investigations on the potential role of the proteinous component of lipofuscin in the pathobiochemistry of various blinding retinal diseases including AMD.

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Table 1
Proteome analysis of lipofuscin in human retinal RPE cells

| Spot | Protein | NCBI | MW (kDa) |
|------|---|----------|----------|
| 1 | Hypothetical protein (alpha-spectrin homolog) | 7512790 | 152.4 |
| 2 | Not identified | | |
| 3 | Tumor rejection antigen (gp96) | 4507677 | 92.7 |
| 4 | Tumor rejection antigen (gp96) | 4507677 | 92.7 |
| 5 | Not identified | | |
| 6 | Hexokinase 1 | 15991833 | 102.2 |
| 7 | Not identified | | |
| 8 | Motor protein | 5803115 | 84.0 |
| 9 | Valosin-containing protein | 6005942 | 90.0 |
| 10 | Heat shock protein HSP 90-alpha (HSP 86) | 123678 | 85.0 |
| 11 | Heat shock 70 kDa protein 5 | 16507237 | 72.3 |
| 12 | Annexin VI (Lipocortin VI) | 113962 | 75.8 |
| 13 | Annexin VI (Lipocortin VI) | 113962 | 75.8 |
| 14 | Annexin VI (Lipocortin VI) | 113962 | 75.8 |
| 14a | Heat shock 70 kDa protein 8 | 5729877 | 70.8 |
| 15 | Calreticulin precursor; Sicca syndrome antigen A | 4757900 | 48.1 |
| 16 | Tubulin, beta chain | 5174739 | 49.6 |
| | Prolyl-4-hydroxylase beta-subunit | 2507460 | 57.0 |
| 17 | Tubulin, alpha-1 chain | 135395 | 50.8 |
| 17a | Tubulin beta chain | 135448 | 50.2 |
| 18 | ATP synthase beta chain | 1145449 | 56.5 |
| 19 | Enolase 2, gamma | 5803011 | 47.6 |
| 20 | Ubiquinol-cytochrome c reductase core protein I | 4507841 | 53.3 |
| 21 | Pyruvate kinase, muscle | 14750405 | 58.5 |
| 22 | Pyruvate kinase, muscle | 14750405 | 58.5 |
| 23 | ATP synthase, alpha subunit | 4757810 | 59.7 |
| 24 | ATP synthase, alpha subunit | 4757810 | 59.7 |
| 25 | Enolase 1, alpha | 4503571 | 47.5 |
| 26 | Enolase 1, alpha | 4503571 | 47.5 |
| 27 | S-arrestin | 14737493 | 45.3 |
| | NADH dehydrogenase Fe-S protein 2 | 4758786 | 52.5 |
| 28 | Glutamate-ammonia ligase | 15297214 | 42.7 |
| | Tu translation elongation factor | 4507733 | 49.5 |
| | NADH dehydrogenase Fe-S protein 2 | 4758786 | 52.5 |
| 29 | Serum albumin | 28592 | 71.3 |
| 30 | Creatin kinase | 180570 | 42.9 |
| 30a | Beta actin | 4501885 | 42.1 |
| 31 | Vimentin | 14742600 | 53.7 |
| 32 | Guanine nucleotide-binding protein (0), alpha subunit 2 | 232134 | 40.6 |
| 33 | Guanine nucleotide-binding protein, beta polypeptide 2 | 4885283 | 38.1 |
| | Pyruvate dehydrogenase | 2144337 | 39.2 |
| 34 | Annexin A2 | 16306978 | 38.8 |
| 35 | Annexin A2 | 16306978 | 38.8 |
| 36 | Annexin A2 | 16306978 | 38.8 |
| 37 | Glyceraldehyde-3-phosphate dehydrogenase | 31645 | 36.2 |
| 38 | Glyceraldehyde-3-phosphate dehydrogenase | 31645 | 36.2 |
| 39 | Glyceraldehyde-3-phosphate dehydrogenase | 31645 | 36.2 |
| 40 | Ubiquinol-cytochrome c reductase core protein II | 14775827 | 48.6 |
| 41 | Porin 31HM (anion channel 1) | 238427 | 30.7 |
| 42 | Voltage-dependent anion channel 2 | 4507881 | 32.1 |
| 43 | Porin 31HM (anion channel 1) | 238427 | 30.7 |
| 44 | Not identified | | |
| 45 | Voltage-dependent anion channel 2 | 4507881 | 32.1 |
| 46 | Glyceraldehyde-3-phosphate dehydrogenase | 31645 | 36.2 |
| 47 | H119n carbonic anhydrase Ii | 2554664 | 29.1 |
| 48 | H119n carbonic anhydrase Ii | 2554664 | 29.1 |
| 48a | Phosphoglycerate mutase 1 | 4505753 | 28.9 |
| 49 | Crystallin, beta B2 | 4503063 | 23.5 |
| 50 | Annexin IV | 14738103 | 27.2 |
| 51 | Annexin V (Lipocortin V) | 999937 | 35.8 |
| | Retinaldehyde binding protein 1 | 4506541 | 36.5 |
| 52 | Tyrosine 3-monooxygenase/tryptophan 5-monoox. activation protein, epsilon | 5803225 | 29.3 |
| 53 | Tyrosine 3-monooxygenase/tryptophan 5-monoox. activation protein, theta | 5803225 | 29.3 |
| | Tyrosine 3-monooxygenase/tryptophan 5-monoox. activation protein, beta | 4507949 | 28.1 |
| | Tyrosine 3-monooxygenase/tryptophan 5-monoox. activation protein, zeta | 4507953 | 27.7 |
| 54 | Cathepsin D | 4503143 | 45.0 |
| 55 | Prohibitin | 4505773 | 29.8 |
| 56 | Prohibitin | 4505773 | 29.8 |
| 56a | Cathepsin D | 4503143 | 45.0 |
| 57 | ATP synthase, subunit d | 5453559 | 18.5 |
| 58 | Recoverin | 4506459 | 23.2 |

Table 1 (Continued).

| Spot | Protein | NCBI | MW (kDa) |
|------|--|----------|----------|
| 59 | Calmodulin 2 (phosphorylase kinase, delta) | 14250065 | 16.8 |
| 60 | ATP synthase, H ⁺ -transporting | 4502297 | 17.4 |
| 61 | Cytochrome c oxidase subunit Va precursor | 4758038 | 16.9 |
| 62 | Peroxiredoxin 2 | 13631440 | 22.0 |
| 63 | Crystallin, alpha A | 4503055 | 20.0 |
| 64 | Crystallin, alpha B | 4503057 | 20.1 |
| 65 | Crystallin, alpha B | 4503057 | 20.1 |
| 66 | Neuropolypeptide h3 | 9131159 | 21.0 |
| 67 | Ubiquinol-cytochrome c reductase binding protein | 5454152 | 13.5 |
| | Hemoglobin alpha 2 | 4504345 | 15.3 |
| 68 | Delta globin | 4504351 | 16.2 |
| 69 | Hemoglobin alpha 1 globin chain | 13195586 | 10.7 |
| 70 | Chain A, P11 (S100a10), ligand of annexin Ii | 3212355 | 11.2 |
| 71 | Mutant hemoglobin beta chain | 18418633 | 16.1 |
| 72 | Mutant hemoglobin beta chain | 18418633 | 16.1 |
| 73 | Mutant hemoglobin beta chain | 18418633 | 16.1 |
| 74 | Cellular retinoic acid binding protein 1 | 18314500 | 15.7 |
| 75 | Cytochrome c oxidase subunit Vib | 4502985 | 10.4 |
| 76 | Glial fibrillary acidic protein | 17479453 | 42.2 |

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