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Proteomics of dedifferentiation of SK-N-BE2 neuroblastoma cells

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ABSTRACT

Neuroblastoma develops through processes which include cellular dedifferentiation. Ability of tumors to form spheroids is one of the manifestations of dedifferentiation and carcinogenic transformation. To study mechanisms of dedifferentiation of neuroblastoma cells, we generated spheroids and performed a proteomics study to compare the spheroids with parental SK-N-BE2 cells. We observed that dedifferentiation induced extensive changes in the proteome profiles of the cells, which affected more than 30% of detected cellular proteins. Using mass spectrometry, we identified 239 proteins affected by dedifferentiation into spheroids as compared to the parental cells. These proteins represented such regulatory processes as transcription, cell cycle regulation, apoptosis, cell adhesion, metabolism, intracellular transport, stress response, and angiogenesis. A number of potent regulators of stemness, differentiation and cancer were detected as subnetworks formed by the identified proteins. Our validation tissue microarray study of 30 neuroblastoma cases confirmed that two of the identified proteins, DISC1 and DNA-PKcs, had their expression increased in advanced malignancies. Thus, our report unveiled extensive changes of the cellular proteome upon dedifferentiation of neuroblastoma cells, indicated top subnetworks and clusters of molecular mechanisms involved in dedifferentiation, and provided candidate biomarkers for clinical studies.

1. Introduction

Acquisition of dedifferentiation features by cells undergoing carcinogenic transformation strongly contributes to tumor growth. Dedifferentiation mechanisms may also contribute to diversity of neuroblastomas [1]. Studies of neuroblastoma show that malignant neuroblastoma cells have properties of cancer stem-like cells and show changes in the differentiation status [2,3]. Existence of stem cells in neuroblastoma cell lines and clinical samples have been reported, and strongly suggests involvement of stemness and dedifferentiation in neuroblastoma carcinogenesis [4,5]. Dedifferentiation is an important key feature in regulation of not only neuroblastoma but also other types of cancers [6]. Knowledge of mechanisms regulating dedifferentiation may lead to a better understanding of neuroblastoma and to identification of novel tumor biomarkers and treatments.

Dedifferentiation can be modeled with cultured cells, and a number of its regulators have been reported. Transcription factors Hif-1α and Hif-2α activate VEGF and glycolysis to induce dedifferentiation and contribute to tumorigenesis [7]. Phox2b and its variants promote dedifferentiation of sympathoadrenergic cells in neuroblastoma by increasing the expression of TLX3 (HOX11) p75 [8]. TLX (NR2E1) is important for neuroblastoma dedifferentiation, thereby promoting tumorigenesis by targeting MMP2 and MMP9 [9]. However, knowledge of mechanisms regulating dedifferentiation is far from complete.

Proteomics is one of the approaches which promises insights into molecular mechanisms governing tumorigenesis. Proteomics-based studies of neuroblastoma has identified complement C3 protein as being highly expressed in the plasma of human neuroblastoma samples [10]. Genomic abberations were reported as important markers in treatment of neuroblastoma [11–14].

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Introduction of cancer systems biology has been successful in detecting otherwise hidden dependencies between different genes and proteins [15,16]. Here, we describe changes of the cellular proteome upon dedifferentiation of neuroblastoma cells. Our results provide insights in regulation of dedifferentiation of neuroblastoma, and show feasibility of the systemic approach for assessing potential neuroblastoma biomarkers.

2. Materials and methods

2.1. Cells and spheroid formation

SK-N-BE2, IMR-32, and SHSY-5Y cell lines were maintained as described previously [9]. Sphere formation was done by culturing the cell lines in tumor-sphere media as described in [9]. Cells were regularly tested for various contaminations, e.g. mycoplasma.

2.2. Proteome profiling

For the analysis of proteome profiling, two-dimensional gel electrophoresis (2-DE), gel image analysis and MALDI-TOF mass spectrometry were used, as described earlier [18]. In brief, 2-DE was performed in IPG dry strips, linear, pH 3-10, 18 cm in an IPGphor instrument (GE Healthcare, Uppsala, Sweden), and in Dalt Six (GE Healthcare) with 10% SDS–PAGE gels. Gels were stained with silver. Protein spots were analyzed using Image Master Platinum Version 6.0 (GE Healthcare). Protein spots which showed difference in expression between two tested conditions (dedifferentiated and differentiated cells) for more than 3-fold of the spot volume, or were unique, were taken for identification by MALDI-TOF mass spectrometry. For each cell line, we generated three 2D gels per condition, i.e. differentiated and dedifferentiated cells. We used replicas of the gels to confirm identification, i.e. proteins were identified independently in separate MS analysis of the same spots excised from different replicas of 2D gels. Statistical significance of reproducibility of spot expression in 2D gels was evaluated by the Image Master 2D Platinum Version 6.0 software. Student's *t*-test was also used to control significance of differences in expression selected spots.

2.3. Protein identification

Protein spots were excised from the gels, destained, and subjected to in-gel digestion with trypsin (modified, sequence grade porcine, Promega, USA), as described earlier [16]. Tryptic peptides were analyzed in a M@LDI R TOF (Micromass/Waters). Peptide spectra were internally calibrated using autolytic peptides from the trypsin (842.51, 1045.56, and 2211.10 Da). To identify proteins, we performed searches in the NCBInr sequence database using the ProFound search engine (http://65.219.84.5/service/prowl/profound.html). One missed cleavage, alkylation with iodoacetamide and partial oxidation of methionine were allowed. Search parameters were set on mass tolerance less than 0.1 Da, no limitations of pI, limits of Mr of +20 and −20 kDa as compared to the migration position of a spot in 2D gel, and "mammalian" was selected for species search. Significance of the identification was evaluated according to the probability value, "*Z*" value, mass precision, number of the matched peptides and sequence coverage.

2.4. Systemic analysis

The GoMiner tool was used to identify functional domains affected by identified proteins. Interactions between the identified proteins from proteomics were analyzed with Cytoscape (http://discover.nci.nih.gov/gominer/). MiMI plug-in was used to analyze related proteins and RNAs from the public databases. The network was viewed in Cytoscape; betweenness was analyzed by Network Analysis, and network modules were extracted by AllegroMCODE tools. Fisher's exact test was used for calculating the *P*-value to determine the network connectivity, and connections with $P < 0.05$ were considered for network. The FunCoup tool (www.funcoup.sbc.su.se) was used to build the small scale networks. Confidence score threshold have been set at 0.50, to ensure significance of the network.

2.5. Immunohistochemistry

MC602 USBiomax (US Biomax, Inc. Rockville, MD, USA) neuroblastoma and peripheral nerve tissue array, 30 cases/60 cores, were used to evaluate expression of DISC1 and DNA-PKcs. The MC602 array contained 25 cases of malignant neuroblastoma from retroperitoneum, abdominal cavity, mediastinum, pelvic cavity, adrenal gland and 5 cases of normal peripheral nerve tissues. Arrays were stained with anti-DISC-1 (H-210, sc-134505) and anti-DNA-PKcs (G-4, sc-5282) from Santa Cruz Biotechnology, Inc., at a dilution of 1:50. Antigen retrieval was performed using Dako-Cytomation target retrieval solution high pH (DAKO, Carpinteria, CA, USA). The slides were stained with Vectastatin Elite ABC Kit (Vector Laboratories Inc., Burlingame, CA, USA) following the manufacturer's instructions, counterstained with hematoxylin and mounted with Flouromount G (Southern Biotechnology, Birmingham, AL, USA). Images of the stained tissues were taken by using a Leica DFC camera, and images were acquired with Leica QWin Standard Software (Leica Microsystems Imaging Solutions Ltd., Cambridge, UK). Intensity of staining was evaluated as absent (−), weak staining in fewer than 5% of cells (+), moderate staining in <50% of cells (++), moderate staining in >50% of cells (+++), and strong staining in >50% of cells (++++). Staining was evaluated in malignant tumor cells. Fisher's exact test was used to calculate the significance of differences in expression of DISC1 and DNA-PKcs.

2.6. Immunoblotting

Cell lysates were resolved on SDS polyacrylamide gels and transferred onto Hybond P membranes (GE Healthcare, Uppsala, Sweden). Membranes were blocked with 5% (v/v) BSA, incubated with a primary antibody overnight at 4 °C, washed with 1 × TBST and after incubation with secondary antibody, the signal was detected by enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden). Antibodies to TLX (Lifespan–LS-B, WA, USA), Oct3/4, Nanog, (Santa Cruz Biotechnology, USA) Sox2 (Abcam), and Nestin (Abcam, Cambridge, England), were used at 1:1000. GAPDH (Sigma, St. Louis, USA) was used at 1:3000, and HRP-conjugated anti-mouse or anti-rabbit IgG was used as secondary antibody at 1:20,000 (Amersham Biosciences, Uppsala, Sweden). Signals were detected by enhanced chemiluminescence (Amersham Biosciences, Uppsala, Sweden).

3. Results

3.1. Neuroblastoma cell lines formed spheres and expressed neural stem cell markers

To mimic dedifferentiation, we generated spheroids formed by neuroblastoma cells, as spheroid formation is considered as the key feature of dedifferentiated stem cells. We cultured neuroblastoma cell lines SK-N-BE2, IMR-32, and SHSY-5Y in the sphere-forming medium, and after two weeks of culturing cells formed spheroids similar to neurospheres (Fig. 1A; data not shown for IMR-32 and SHSY-5Y). We further investigated the stem-cell like

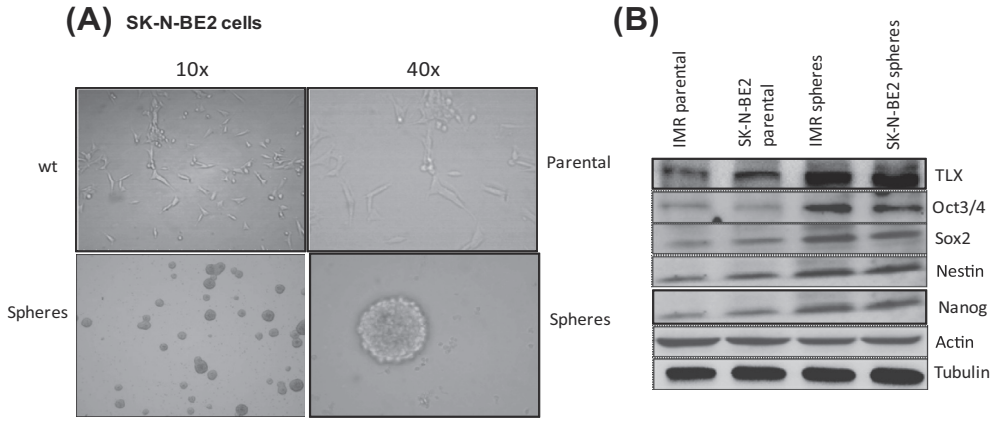


Fig. 1. Neuroblastoma spheroids expresses neural stem cell markers. (A) Neuroblastoma cell line SK-N-BE2 spheres cultured under dedifferentiated condition, forming spheroids as compared with the parental cells. Phase contrast microscopic pictures taken by10× and 40× magnification, as indicated. (B) Immunoblotting for neural stem cell markers TLX, Oct3/4, Sox2, Nestin, and Nanog in SK-N-BE2 and IMR-32 spheres, and the parental cells. Immunoblotting for actin and tubulin were used as loading controls.

features by immunoblotting to detect different neural stem cell markers. We observed elevated expression of TLX, Oc3/4, Sox2, Nestin, and Nanog in neuroblastoma spheres compared with parental cells (Fig. 1B). Our results are in agreement with reports that SK-N-BE2 cells contain cells with markers of stemness and may form spheres [5,9]. We observed formation of spheres already after 1 week of culturing. However, we continued for one more week to form spheres of size from 100 to 250 μM in diameter. No apoptotic bodies or cell debris were observed in the sheres culture, which indicated no extensive cell death. No changes in expression of actin and tubulin in spheres as compared to the parental cells also suggested lack of extensive cell death (Fig. 1B). Thus, expression of the markers confirmed the dedifferentiation status and acquisition of the stem cell-like features by the cells in spheres.

3.2. Protein expression maps of neuroblastoma cell lines and spheres

To generate protein expression maps, we focused on studying intact proteins. We generated 2D gels for SK-N-BE2, IMR-32 and SHSY-5Y parental cells and spheroids. In average, for each parental cell line from 1300 to 1600 protein spots were detected, and for each spheroid culture from 1100 to 1400 protein spots were detected in 2D gels (Fig. 2A and B; data not shown for IMR-32 and SHSY-5Y). We observed a significant variability in the patterns of separated proteins in 2D gels between the dedifferentiated cells in spheroids and cells cultured on a substrate at standard culturing conditions.

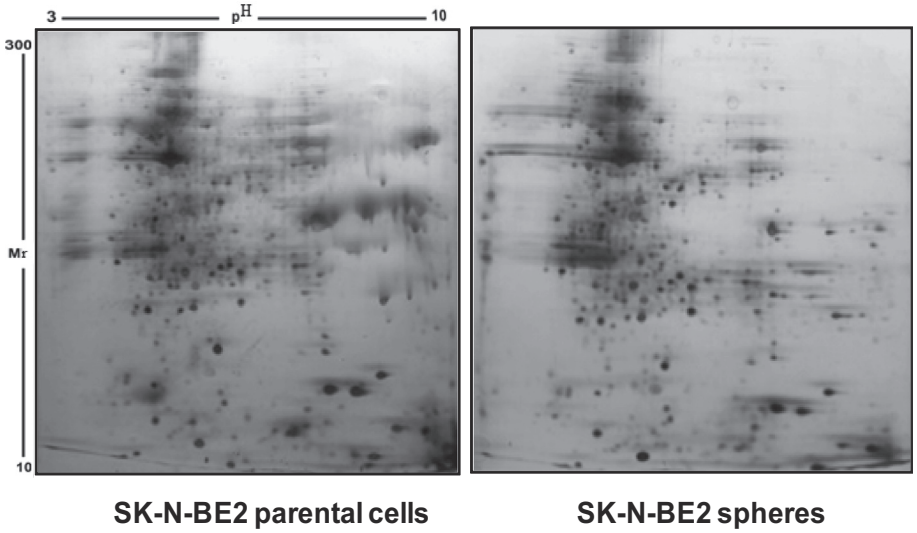
We proceeded to identification of selected proteins for SK-N-BE2 cells, since the overall patterns of observed proteome changes were similar for all 3 studied cell lines. Therefore, only protein spots in SK-N-BE2 cells were selected to identification. The variability in proteome expression maps between the parental and spheroids led to detection of only 533 common spots between parental and spheres of SK-N-BE2 cells. A total of 330 protein spots, being unique or having changed their expression more than 3-fold were selected for mass spectrometry-based identification, and 239 proteins were identified unambiguously (Fig. 2 and Supplementary Figs. 1 and 2). We repeated identifications of proteins in the same spots in different gels to validate identifications. Among identified proteins were Nanog (spot 239), metastasis associated 1 (spot 60), Sox2 (spot 221), and glial cell-derived neurotrophic factor (spot 74). These proteins are known to be involved in regulation of differentiation, stemness and metastasis, and their identification confirmed biological relevance of our observation. It has also to be noted that the variability in numbers of detected protein spots between gels of the same condition was less than 10%. This suggests that the observed high variability between parental cells and spheres was of biological relevance, and not a technical issue. The significant variability between the parental cells and spheroids suggests that dedifferentiation may lead to extensive changes in the pattern of protein expression in cells.

3.3. Systemic analysis of spheres-associated proteome signature

To investigate the biological functions of the identified proteins from SK-N-BE2 spheres, we performed analysis of functions represented by the identified proteins and relations between these proteins. We used GoMiner analysis to identify functional clusters represented by the identified proteins. This analysis showed that the major functions of the proteins were regulation of development, transcription, neurogenesis and transport. It also included regulation of the cell cycle, cell death, cell division, cell homeostasis, cell organization, cell proliferation, metabolism and signaling. Thus, the significant difference between protomes of cells in spheres and of parental cells and the broad functional importance of the affected domains show that the dedifferentiation is associated with extensive changes of the cellular proteome and consequently the cell physiology.

To explore relations between the identified 129 upregulated proteins, we generated networks of interactions between these proteins and the proteins and genes they may affect (Fig. 3). We observed that the distance between the nodes and connectivities were such that the network formed a tight structure, with exception of Fc region receptor III A (FCGR3, also known as CD16a) – related subnetwork. Many of the identified proteins were in dense areas of the network, i.e. close to the central nodes. The nodes of high importance for robustness of the network are often the nodes of highest weight in the network and the highest centrality parameters such as betweenness [17]. These nodes represent key regulators, as they have the strongest impact on the network stability and response to perturbation. The keynodes defined as regulators of stemness would also be crucial regulators of the tumorigenesis and development. These following nodes were identified as potential key regulators of the dedifferentiation: TAF1, HELLS, ELAV2, LY6H, NRTN, TRH, CNNM4 and GH1 (Fig. 3B). The impact of other nodes, for example NRTN was estimated to be significant due to high betweenness. Important feature of the network is high level of node connectivity, as there is a large number of nodes with high connectivity (Node Degree values; Fig. 3B). These nodes may also

(A)



(B)

Common Spots for parental cells and spheres	Spots for SK-N-BE2 parental cells	Spots for SK-N-BE2 spheres
533 Total **129 up-regulated** **80 down -regulated**	1380	1190

Fig. 2. Proteome profiling. (A) Representative images of 2D gels obtained with proteins extracted from SK-N-BE2 parental cells or spheres are shown. Directions of isoelectrofocusing and SDS–PAGE are indicated. Migration positions of proteins are annotated in Supplementary Fig. 1. (B) Numbers of identified proteins observed as unique, common, up- and down-regulated in spheres, as compared to the parental cells are indicated in the first column. The second and the third columns indicate number of protein spots detected by the image analysis program in 2D gels of the indicated cells.

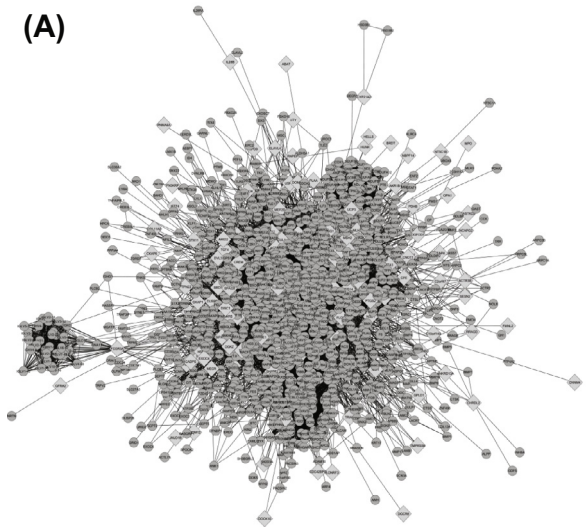
represent key regulators of dedifferentiation. Thus, analysis of topology of the network allowed identification of keynodes of importance for regulation of dedifferentiation.

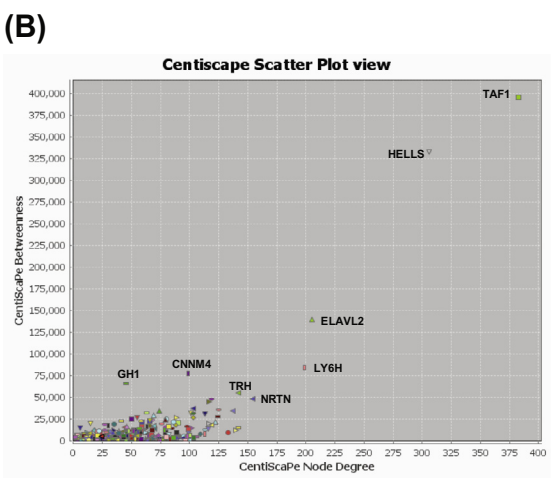
To elaborate on identification of the key regulators of dedifferentiation in an alternative way, we analyzed the generated network for presence of subnetworks. Subnetworks represent nodes with higher connectivity between them, as compared to other nodes in the whole network. In biological terms, detection of subnetworks allows to outline specific regulatory processes integrated in the whole network. We observed a number of subnetworks in the whole network (Fig. 3). The top ranked subnetworks represented regulation of protein degradation, cell apoptosis, cell proliferation, cell death, and cell-cycle regulation. The subnetwork analysis showed involvement in dedifferentiation and stemness of many known regulators, such as CNOT1, Sox2, Nanog, AMIGO1, DISC1, DARS, RAE1, MSTO1, and CDC25B (Fig. 3). This analysis identified also regulators that have not been previously associated with stemness and cancer, such as DISC1 and DNA-PKcs.

We focused on analysis of dedifferentiation-related subnetworks, and explored links of our identified proteins with such functions as differentiation, stemness and cancer, since these functions are important for neuroblastoma development. By using database searches with our data, we assigned 18 proteins in group "Stemness", 27 proteins in "Differentiation", and 19 proteins in "Cancer" (Fig. 3C). Networks were built for each category to characterize regulators related to these categories, and the subnetworks for "Stemness", "Cancer" and "Differentiation" are shown in Fig. 3D–F. It is established that the key cellular functions may be regulated in the same manner by different components of a given signaling network [17]. The subnetworks (Fig. 3D–F) show that even selected function, i.e. stemness, cancer and differentiation, involve cross-connections between the regulators. Therefore, it is expected that the whole network representing all functions (Fig. 3A) is very compact and has extensive cross-connectivity of the nodes. Analysis of networks and subnetworks proved to be an efficient tool to gain insights in regulatory mechanisms engaged in the dedifferentiation and tumorigenesis. Thus, our systemic analysis contributed to the list of regulators of dedifferentiation of neuroblastoma cells, and indicated relations between these regulators (Fig. 3).

3.4. Validation of DISC1 and DNA-PKc expression

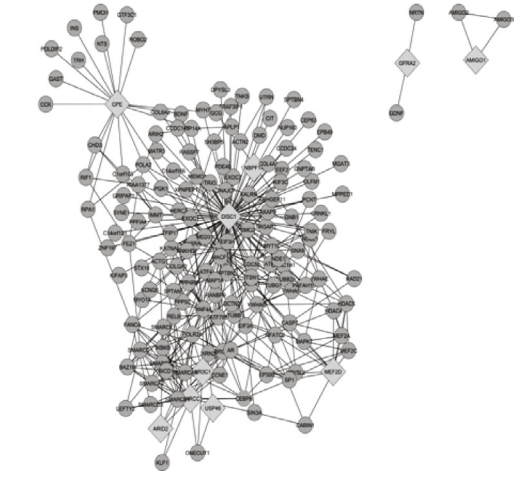
To validate our proteomics data by an alternative technique, we explored expression of DISC1 and DNA-PKcs by immunohistochemistry in tissue microarray cases. DISC1 and DNA-PKcs proteins were selected due to their positioning in the network, and also due to previous reports suggesting that these proteins may have a potential role in tumorigenesis [18,19]. We used tissue microarray of neuroblastoma specimens, and 30 cases were analyzed. We observed an increase in the expression of DISC1 and DNA-PKcs in the tumor tissues (Fig. 4A–D; Supplementary Fig. 3). MC602 tissue microarrays showed strong DISC1 staining (++++) and DNA-PKcs

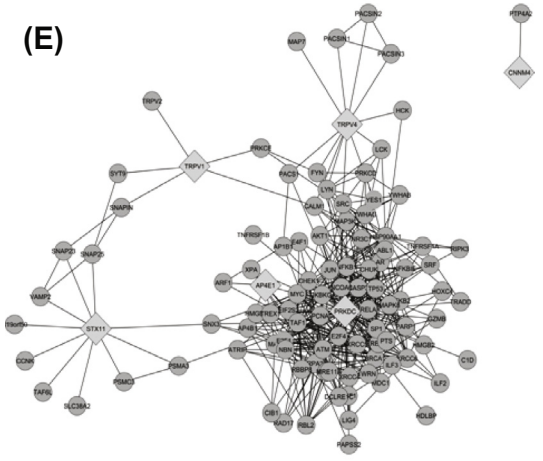




(C)

Stemness	Differentiation	Cancer
CNOT1	BRDT	JMJD1B
PRDM12	TRPV4	NR3C1
Sox2	TRPV1	MAK
Nanog	MEF2D	NR2C1
SMARCC1	GFRA2	ELL
HNT	PAPLN	CPE
AMIGO1	NIN	MLH1
DISC1	SIPA1L1	FBXO4
DARS	LRP1	NID1
CDC25B	DGKZ	FOLH1
COL19A1	DOCK10	EIF3S6
COBL	HSPH1	MTA3
MSTO1	BRD1	CAMSAP1L1
RPUSD2	RPSUD2	ARID2
RAE1	DPP9	TES
DOM3Z	TMPRSS6	FMNL2
DLG2	IL28B	GPLD1
HEART3	MSTO1	TFAP4
	EGFR	PRKDC
	CAPN5	
	MYD88	
	MSH2	
	DDX34	
	PTGS2	
	SERPINB3	
	FCGR3	
	GDPD5	





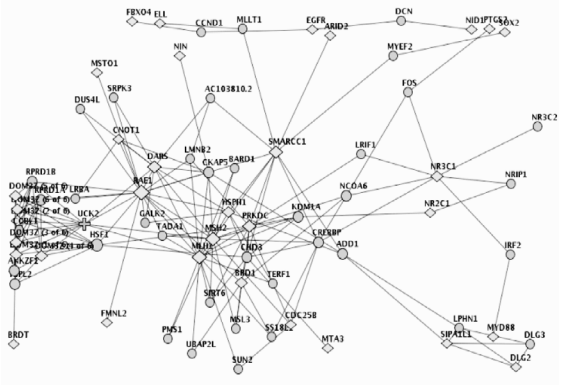


Fig. 3. Network formed by the identified proteins. (A) Network formed by the identified proteins is shown. Cytoscape was used to build a network by using AllegroMCODE tool. Diamonds represent proteins identified by proteome profiling, and cycles show networks components which interact with the identified proteins. The cycle-annotated components were defined as "neighbours of neighbours of the identified proteins" upon building the network. The components may be proteins and/or genes, and are included in the network after Cytoscape searched for physical or/and functional interactions of the identified proteins. (B) Betweenness and centrality of nodes of the network are presented. The nodes are plotted as functions of degree connectivity and betweenness. Some of the nodes with standing out values are annotated in Gene Ontology terms. (C) Proteins annotated to categories Stemness, Differentiation and Cancer are listed. Proteins are annotated in Gene Ontology terms. The proteins of these categories were extracted from the network shown in panel A. Subnetworks formed by the proteins assigned to the categories "Stemness" (D), "Cancer" (E) and "Differentiation" (F) are shown. Diamonds indicate proteins identified by the proteome profiling, and circles indicate nodes incorporated by the systemic analysis. These nodes may be genes or proteins which interact physically or functionally with the identified proteins.

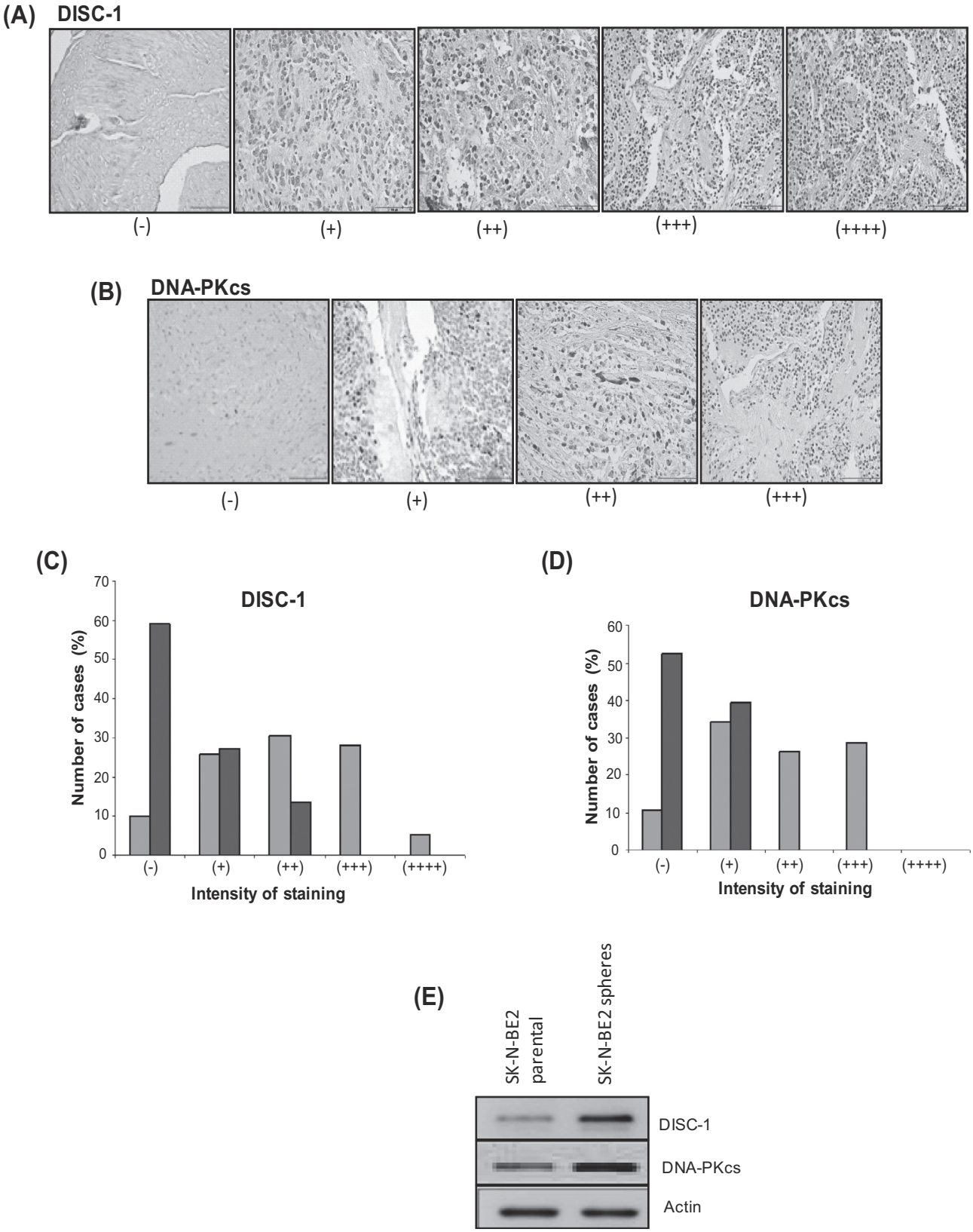


Fig. 4. Expression of DISC1 and DNA-PKcs in neuroblastoma. Immunohistochemistry images of detection of DISC1 (A) and DNA-PKcs (B) in tumor and histologically normal tissues of the tissue microarray. Representative images are shown (scale bar represents 100 μM). Quantification of staining of tumor (grey) and normal (black) tissues is shown for DISC1 (C) and DNA-PKcs (D). (E) Immunoblot analysis of DISC1 and DNA-PKcs expression in parental SK-N-BE2 and SK-N-BE2 spheres. Actin was used as a loading control.

showed slightly weaker staining (+++). Tumor cells consistently showed staining for DISC1 in a lower number of cells and of weaker intensity. A similar observation was made for DNA-PKcs. We did not observe significant differences in intracellular nuclear vs cytoplasmic distribution of the staining signals. Thus, our TMA results validated our proteomics data of DISC1 and DNA-PKcs expression, and opened for a full-scale biomarker study which would require large groups of cases. Such a full-scale biomarker study would also be a follow-up study for clinical implementation of DISC1 and DNA-PKcs.

We also validated these two proteins on the same SK-N-BE2 parental and spheres by immunoblotting, and observed elevated levels of expression of DISC1 and DNA-PKcs in spheres compared with wild type (Fig. 4E). Furthermore, we also performed searches of publicly available data from http://hgserver1.amc.nl/cgi-bin/r2/main.cgi. We found the gene microarray data from 88 clinical samples of Neuroblastoma-Versteeg MAS5.0 – u133p2, which analyzed DISC1 and DNA-PKcs. A Kaplan-Meier analysis indicated that the higher expression of DISC1 and DNA-PKcs correlated with patients' poor survival rate with statistical significance of $p = 0{,}015$ but Bonferroni $p = 1.00$ for DISC1, and $p = 1.7e\text{-}10$ and Bonferroni $p = 1.2e\text{-}08$ for DNA-PKcs. Our data about DISC1 and DNA-PKcs are in lane with the reported observations, and call for further studies. Thus, our immunohistochemistry, immunoblotting and literature studies confirmed our proteomics observations, and provide the basis for further clinical studies of the identified proteins as biomarkers with large cohorts of patients.

4. Discussion

There have been a number of attempts to explore proteome changes in neuroblastoma cells subjected to different treatments, e.g. with curcumin or cisplatin, and in high and low risk tumors or regressing tumors [20–23]. There was also a report of identification of proteins differentially expressed in the human NB cells cultured in 2D culture and in spheroids [24]. In the NB cells model, proteins involved in regulation of metabolism, stress response, cell structure, signal transduction, biosynthesis and transport were identified. However, lack of a systemic analysis restricted exploration of these proteomics results. All reported proteomics studies were limited to identification and validation of differentially expressed proteins. There have been no reports of a comprehensive systems biology study of neuroblastoma.

As current proteomics technologies are not capable to cover the full human proteome, it is important to use advanced systems biology to decipher molecular profile of changes detected by proteomics. Our study has successfully combined proteomics and systems biology in gaining the insight into complexity of mechanisms engaged in dedifferentiation. The change in expression of more than 30% out of more than 1500 detected proteins, and involvement of many crucial cellular functions (Figs. 2 and 3) is a strong indication of an extensive intracellular re-arrangements. The list of the identified by us proteins provides a source for further in-depth studies.

Our validation study of expression of DISC1 and DNA-PKcs (Fig. 4) confirmed the proteomics results. We used immunohistochemistry and immunoblotting to confirm that these proteins showed increased expression in malignant tissues. Moreover, search with DISC1 and DNA-PKcs in available datasets of survival of patients showed that enhanced expressions of DISC1 and DNA-PKcs indeed may correlate with poor survival. To develop DISC1 and DNA-PKcs as a predictive markers, a separate clinical study of DISC1 and DNA-PKcs expression in a large number of malignant and normal tissues have to be performed. Our observations (Fig. 4) provide encouraging rationale supporting such a large scale clinical study.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.065.

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