ISSN 1990-519X, Cell and Tissue Biology, 2018, Vol. 12, No. 6, pp. 455–459. © Pleiades Publishing, Ltd., 2018.
Original Russian Text © E.S. Petrenko, A.T. Kopylov, O.A. Kleist, O.K. Legina, N.V. Belyakova, R.A. Pantina, S.N. Naryzhny, 2018, published in Tsitologiya, 2018, Vol. 60, No. 7, pp. 519–523.

Searching for Specific Markers of Glioblastoma: Analysis of Glioblastoma Cell Proteoforms

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Received March 3, 2018

Abstract—Using the sectional analysis of two-dimensional electrophoretic gels with liquid chromatography-mass spectrometry, proteoform profiles for individual genes expressed in cancer (glioblastoma) and normal (FLEH) cells were obtained. Profiles of more than 5000 genes were analyzed. It turned out that many genes encoding potential biomarkers of glioblastoma are characterized by sets of proteoforms that are different in normal and cancer cells. These proteoforms could be sources of highly specific markers and targets for therapy. Using a section analysis of two-dimensional electrophoretic gels with liquid chromatography by mass spectrometry, proteoform profiles were obtained for individual genes expressed in cancer (glioblastoma) and normal (FLEH) cells. Profiles of more than 5000 genes were analyzed. It turned out that many genes encoding potential biomarkers of glioblastoma are characterized by sets of proteoforms, which are different in normal and cancer cells. These proteoforms could be sources of highly specific markers and targets for therapy.

Keywords: glioblastoma, proteome, biomarker, proteoform, two-dimensional electrophoresis, mass spectrometry

DOI: 10.1134/S1990519X18060068

INTRODUCTION

Glioblastoma is the most common and most aggressive form of brain tumor, which accounts for up to 52% of primary brain tumors and up to 20% of all intracranial tumors. The average life expectancy of patients with glioblastoma is about 1 year that emphasizes the need for early diagnosis and finding ways to treat the tumor (Furnari et al., 2007; Louis et al. 2007). Currently, the main methods of diagnostic of this disease are computer tomography and brain biopsy (Fiorentino et al., 2013). Therefore, there is an urgent need to develop new noninvasive methods for early diagnosis. Determination of the proteome profile by two-dimensional electrophoresis (2DE) in combination with mass spectrometry and immunodetection allows one to identify individual proteins and observe a change in their content and composition of proteoforms in normal and pathological conditions, as well as under the influence of various factors, that will enable them to be included in the list of potential candidates for use in the diagnosis and treatment of this disease (Zhang et al., 2003; Cohen and Colman 2015; Ludwig and Kornblum 2017; Kalinina et al., 2011). We used normal (human lung fibroblasts, FLEH) and cancer (glioblastoma) cell lines as the analyzed material. It would seem that this is not an ideal pair for comparison (normal and cancer lines), however previously we analyzed the proteomic profiles of these cells and showed that they are very similar (Naryzhny et al., 2014a, Naryzhny et al., 2016b). The levels of some proteins in these cells vary greatly (Naryzhny et al., 2014a, Naryzhny et al., 2016b). Alpha-enolase (ENOA_HUMAN), annexin 1 (ANXA1_HUMAN), annexin 2 (ANXA2_HUMAN), PCNA (PCNA_HUMAN), p53 (TP53_HUMAN), and others are among these proteins. With the help of our research methods, we had the opportunity to conduct a more detailed panoramic analysis of not just proteins but also proteoforms (specific protein forms). Construction of 3D-graphs, where each graph quantitatively reflects a set of proteoforms, encoded by the same gene, allowed to visualize all their variety. Panoramic analysis of these graphs showed that many potential biomarkers of glioblastoma have proteoforms, absent in normal cells. These proteoforms could become sources of highly specific markers and targets for therapy.

Abbreviations: IEF—isoelectric focusing, FLEH—fibroblasts of lungs of human embryos, 2DE—two-dimensional electrophoresis, ESI LC-MS/MS—liquid chromatography–mass spectrometry.

MATERIALS AND METHODS

All reagents used were obtained from Sigma-Aldrich (United States), unless specified by another manufacturer. Other reagents were dithiothreitol (DTT) and protease inhibitor cocktails (Pierce, United States); CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate, IPG-gel strips, IPG buffers, DryStrip-coating liquid (GE Healthcare, United States); Trypsin Gold (Promega, United States); methanol, acetic acid, Coomassie R350 reagents (Fisher Scientific, United States); Tris, 10X TGS-buffer (Tris, glycine, SDS), markers of Protein mol. weight for electrophoresis (Bio-Rad Laboratories, United States); RPMI-1640 and DMEM media for cell growth, fetal bovine serum (Biolot, Russia); Fetal Clone II serum (HyClone, United States); and Carrel culture bottles (Orange Scientific, Belgium).

Cell Cultures and Cultivation Conditions

Convoluted human FLEH (human embryonic lung fibroblast) cell cultures, L-glioma cells (glial primary lines obtained at the Petersburg Nuclear Physics Institute, Gatchina) were cultured in DMEM or RPMI-1640 medium containing 5% fetal serum, without antibiotics, in a 5% CO_2 atmosphere at 37°C.

Two-Dimensional Electrophoresis (2DE)

Samples were prepared as described earlier (Naryzhny, 2009; Naryzhny et al., 2014b). Cells (~10^7) containing 2 mg protein was treated with 100 μL of lysing buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% dithiothreitol (DTT), 2% ampholytes, pH 3–10, a mixture of protease inhibitors). The protein concentration in the sample was determined by the Bradford method (Bradford, 1976). Proteins were separated by isoelectrofocusing (IEF) using 7-cm Immobiline DryStrip strips, pH 3–11 (GE Healthcare, United States) following the manufacturer's protocol. Samples were mixed with rehydrating buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.3% DTT, 0.5% IPG buffer, pH 3–11, 0.001% Bromphenol blue) in a 130-μL final volume (150-μg protein). The strips were passively rehydrated for 4 hours at 4°C. IEF was conducted on a 3100 OFF GEL Fractionator (Agilent Technologies, United States), which was programmed to receive 10000 volt hours for 14 h at 20°C. After IEF, the strips were equilibrated for 10 min in an equilibration buffer (50 mM Tris pH 6.8, 6 M urea, 2% SDS and 30% glycerol) containing 1% DTT. This process was repeated with a counterbalancing buffer that contained 5% (w/v) iodoacetamide instead of DTT. The strips were placed on top of a 12% polyacrylamide gel of the second direction and sealed with 1 mL of a 0.5% agarose hot solution in electrode buffer (25 mM Tris, pH 8.3, 200 mM glycine, and 0.1% SDS) and electrophoresed the second direction using a Hoefer miniVE system (gel size 80 × 90 ×1 mm), with a constant power of 3 W per gel (Naryzhny et al., 2017).

Mass Spectrometry Sectional Analysis

All procedures were performed according to the previously described protocol (Naryzhny et al., 2016b, 2017). After 2DE separation and Coomassie R350 staining, the gel was scanned, calibrated, and divided into 96 sections with predetermined coordinates. Each section (~0.7 cm^2) was cut into small parts and treated with trypsin according to a protocol for the treatment of individual spots. The trypsinolysis-derived peptides were extracted with a solution (5% (v/v) acetonitrile (ACN), 5% (v/v) formic acid) and dried in a SpeedVac vacuum centrifuge (Thermo Fisher Scientific, United States).

For analysis, the peptides were dissolved in 20 μL of 5% (v/v) formic acid. Liquid chromatography mass spectrometry (ESI LC-MS/MS) of the obtained peptides was carried out using an Agilent HPLC Series 1100 chromatography system (Agilent Technologies, United States). Approximately 4 μg of peptides were added to a Zorbax 300SB-C18 5 × 0.3-mm trap column (Agilent Technologies). After washing with a 5% ACN solution containing 0.1% formic acid, the peptides were separated on a 150 mm × 75-μm backphase analytical Zorbax 300SB-C18 (Agilent Technologies) column by a 30-minute gradient (5–60% ACN, 0.1% formic acid) at a flow rate of 300 nL/min. Tandem MS/MS analysis was performed in duplicate on an Orbitrap Q-Exactive (Thermo Scientific) mass spectrometer (Naryzhny et al., 2016c, 2017). Mass spectra were collected in a positive ion mode. High-resolution data was obtained on the Orbitrap analyzer with a resolution of 30000 (m/z 400) for MS scans and 7500 (m/z 400) for MS/MS scans. Data was analyzed by Mascot 2.4.1 (www.matrixscience.com) using the following parameters: enzyme, trypsin; maximum of missed cleavage sites, 2; fixed modifications, carbaidomethylation of cysteine; variable modifications, oxidation of methionine, phosphorylation of serine, threonine, tryptophan, acetylation of lysine; the range of the precursor mass error, 20 ppm; the product mass error, 0.01 Da. The sequence search was carried out in the NexProt database. For quantitative evaluation, the emPAI parameter (exponentially modified protein abundance index) was used, which is defined as the number of peptides identified divided by the number of theoretically possible peptides for each protein (Ishihama et al., 2005).

RESULTS AND DISCUSSION

To obtain a more detailed picture of the proteoforms present in normal and cancer cells, we conducted a sectional analysis of two-dimensional electrophoretic gels (Naryzhny et al., 2016b). To do this, the cellular proteins were separated with 2DE, the gels

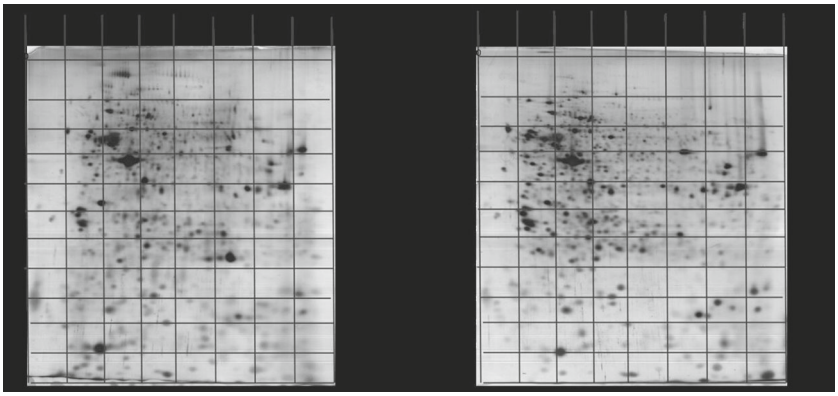

Fig. 1. 2DE maps of the extract of normal FLEH cells (left) and glioblastoma (right). On the X axis—*pI* isoelectric point; on the Y axis—mol. weight. 500 mg of protein was applied. The gel size was 80 × 90 × 1 mm. The sections of the gel chosen for the subsequent mass spectrometric analysis are shown.

were stained with Coomassie, and two-dimensional protein patterns characteristic of each cell line were obtained (Fig. 1). Based on the previously obtained information (isoelectric point, pI/mass, Mw), the most characteristic detected proteins of the map were normalized by isoelectric point and mass. Then, gels of 7 × 8-cm size were divided into 96 sections with predetermined coordinate ranges (pI/Mw) and each section was trypsinized to obtain specific peptides, which were then analyzed using ESI LC-MS/MS. As a result, from 140 to 600 proteins (proteoform) were identified in each section. We considered the presence of the same protein in different sections as its existence in different proteoforms. Proceeding from this principle, we identified 29 180 proteoforms (products of 5159 genes) in normal and 27 851 proteoforms (products of 5841 genes) in glioblastoma cells. For each gene, tables of the corresponding proteoforms were made and three-dimensional graphs of their distribution on a two-dimensional gel were constructed. The products of 3340 genes (proteins) were identified in both lines. The fact that some proteins were detected only in one of the lines (1819 in FLEH and 2501 in glioblastoma cells only) is most likely associated with both their level and sensitivity of detection. For example, the p53 protein was detected in the form of only one proteoform and only in glioblastoma cells (data not shown). However, using enzyme immunoassay (Western blot), p53 is well detected by us in both glioblastoma (up to 30 proteoforms) and normal (up to 6 proteoforms) cells (Naryzhny et al., 2014b).

We paid special attention to proteins that, earlier on the basis of their data and from literature, were selected as potential glioblastoma biomarkers (Naryzhny et al., 2014a; Naryzhny et al., 2016b). We performed a comparative analysis of proteoform profiles of these proteins, which are partially represented in Fig. 2. As in the case of protein profiles, proteoform profiles are most often very similar, but in some cases there are also peculiarities. Thus, in glioblastoma cells, unlike FLEH cells, ANXA1, ANXA2, KPYM, and HSPB1 proteins have proteoforms with a more alkaline isoelectric point (Fig. 2). It should be noted that a shift to the alkaline region is possible with modifications of carboxyl groups (amidation, esterification). In addition, the profiles of some proteins, such as ANXA1 and ANXA2, in cancer cells have a more pronounced heterogeneity (many proteoforms of approximately the same level). The same feature was observed earlier for the p53 protein, where we used immunodetection (Western blot analysis) (Naryzhny et al., 2014a). It should be noted that we consider the obtained data as a starting point for a more detailed analysis of the detected proteoforms. Later, it will be necessary to confirm the results with immunodetection (Western blot analysis), to more accurately determine the parameters (pI/Mw) and the amino acid sequence of the proteoforms, including the available posttranslational modifications. Given that the section analysis that we used still has a very weak resolution, in the future there is an opportunity for a maximization of the 2DE potential and more accurate assessment of the proteoforms of interest.

ACKNOWLEDGMENTS

This work was performed with support from the Russian Foundation for Basic Research of State Academies of Sciences for 2013–2020. Mass-spectrometry measurements were performed using the equipment of "Human Proteome" Core Facilities of the Institute of Biomedical Chemistry (Russia) which is supported by Ministry of Education and Science of the Russian Federation (agreement 14.621.21.0017, unique project ID RFMEFI62117X0017).

COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interests. The authors declare that they have no conflict of interest.

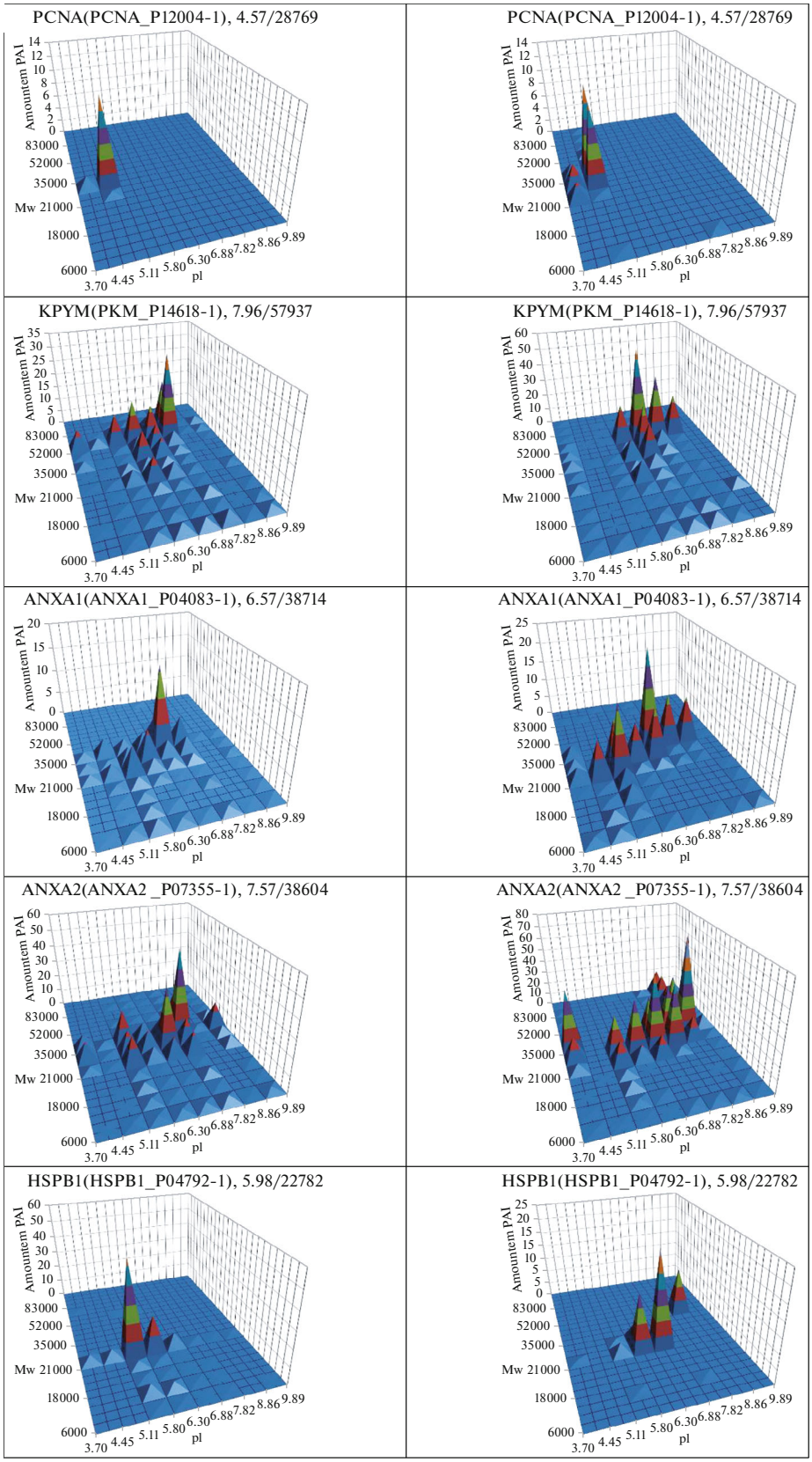

Fig. 2. Profiles of some proteins detected in normal (FLEH) and cancer (glioblastoma) cell lines. The column on the left—fibroblasts, on the right—glioblastoma. The name of the protein is given above each histogram, the Uniprot number, and the theoretical values of pI/Mw. Coordinates: isoelectric point (pI), molecular weight (Mw), protein amount (emPAI).

Statement on the welfare of animals. This article does not contain any studies involving animals performed by any of the authors.

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Translated by P. Kuchina