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VARIABILITY OF HAPTOGLOBIN BETA-CHAIN PROTEOFORMS

N.L. Ronzhina[1], E.S. Zorina[2], M.G. Zavialova[2], O.K. Legina[1], S.N. Naryzhny[1]*

[1]B.P. Konstantinov Petersburg Institute of Nuclear Physics, National Research Center "Kurchatov Institute", 1 Orlova Roshcha, Gatchina, Leningrad Region, 188300 Russia; *e-mail: snaryzhny@mail.ru
[2]Institute of Biomedical Chemistry, 10 Pogodinskaya str., Moscow, 119121 Russia

Existing knowledge on changes of the haptoglobin (Hp) molecule suggests that it may exist in multiple proteoforms, which obviously exhibit different functions. Using two-dimensional electrophoresis (2DE) in combination with mass spectrometry and immunodetection, we have analyzed blood plasma samples from both healthy donors and patients with primary grade IV glioblastoma (GBM), and obtained a detailed composite 2DE distribution map of β-chain proteoforms, as well as the full-length form of Hp (zonulin). Although the total level of plasma Hp exceeded normal values in cancer patients (especially patients with GBM), the presence of particuar proteoforms, detected by their position on the 2DE map, was very individual. Variability was found in both zonulin and the Hp β-chain. The presence of an alkaline form of zonulin in plasma can be considered a conditional, but insufficient, GBM biomarker. In other words, we found that at the level of minor proteoforms of Hp, even in normal conditions, there was a high individual variability. On the one hand, this raises questions about the reasons for such variability, if it is present not only in Hp, but also in other proteins. On the other hand, this may explain the discrepancy between the number of experimentally detected proteoforms and the theoretically possible ones not only in Hp, but also in other proteins.

Key words: haptoglobin; proteoforms; glioblastoma; individual variability

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INTRODUCTION

Haptoglobin (Hp), one of the major blood proteins, is an acute phase protein that binds hemoglobin thus preventing oxidative damage. Good evidence exists that Hp may be a promising biomarker for diseases. This idea is confirmed by a number of publications that discuss the possible role of Hp as a tumor marker [1–6]. Hp is one of the major plasma proteins (0.38–2.08 g/l), being an acute phase protein, its main function consists in binding of free hemoglobin [7, 8]. Hp consists of two polypeptide chains (α and β) covalently linked by disulfide bonds. Both chains are encoded by one gene located on chromosome 16 [9, 10]. Only in humans, the Hp gene is polymorphic, has three structural alleles that control synthesis of three major phenotypes of Hp, homozygous Hp1-1 and Hp2-2, and heterozygous Hp2-1, determined by a combination of allelic variants (α1 or α2) that are inherited [11]. In addition, Hp undergoes various post-translational modifications (PTMs). These include structural transformations (removal of the signal peptide, cutting of the Pre-Hp precursor molecule into two subunits, α and β, limited proteolysis of the α chains, formation of disulfide bonds, multimerization), as well as chemical modifications of the α and β chains. A particularly large number of protein variants (proteoforms) can be formed during glycosylation of the Hp β chain at four Asn sites (Asn184, Asn207, Asn211, Asn241) [12, 13]. Using two-dimensional electrophoresis (2DE), these proteoforms are detected as a chain of at least 10 spots. Proteoforms of the Hp α1-chain and α2-chain migrate in at least three spots with approximately the same mass (~9 kDa or ~16 kDa, respectively), but with different p*I* [14]. In some cases, the Pre-Hp precursor (more precisely, only with the Hp2-2 phenotype) can function under the name zonulin as a single polypeptide that performs functions different from the classical functions of Hp [15]. Zonulin is a protein involved in the regulation of the tight junctions between epithelial cells. The level of blood zonulin reflects intestinal permeability, and its elevated level is considered an indicator of the impaired intestinal barrier function [15]. The results we have obtained from a comparative analysis of plasma samples from healthy people and patients with glioblastoma (GBM) indicate that Hp and its proteoforms could be used as prognostic biomarkers of GBM [16].

We previously examined biological samples by 2DE, followed by gel cutting into sections and subsequent mass spectrometry (LC-ESI-MS/MS) analysis of the proteins contained in each section [17]. Using such shotgun analysis it is possible to detect all the proteins present in the gel (not just those visualized as spots after gel staining). Despite a loss of resolution (each 1 cm × 0.5 cm section may contain multiple proteoforms of the same protein), the resulting typical section distribution pattern indicates the presence of a much larger number of Hp proteoforms than previously known [18]. In addition, our previous data indicate the possibility of the existence of specific plasma proteoforms that are present only in GBM, for example, the full-length, unprocessed form of Hp known as zonulin [16].

We have continued research in this field. The aim of this work was, firstly, to obtain statistically significant confirmation of the presence in plasma samples of GBM patients of an unusual proteoform of zonulin with a p*I* value shifted to the alkaline region; secondly, to study the possible presence of specific proteoforms of the Hp β-chain in the plasma of GBM patients. To clarify and confirm this situation, we have conducted a detailed analysis of the plasma samples available. To obtain further information on Hp proteoforms, we have performed comparative proteomic analysis of several plasma samples based on protein separation using 2DE.

MATERIALS AND METHODS

All reagents used were obtained from Sigma-Aldrich (USA), unless another manufacturer was indicated. The remaining reagents were from the following companies: dithiothriitol (DTT) and a mixture of protease inhibitors from Thermo Fisher Scientific (USA); IPG DryStrip (gel strips), IPG buffers, DryStrip-coating liquid, Coomassie R350, acrylamide solution with bisacrylamide (PlusOne ReadySol IEF 40% T, 3% C) from GE Healthcare (USA); Trypsin Gold from Promega (USA); protein molecular weight markers for electrophoresis from Bio-Rad (USA). All patients with glioma included in the study were diagnosed with grade IV primary GBM. In addition, blood samples from non-GBM patients and healthy donors were obtained from the Pavlov First St. Petersburg State Medical University. Venous blood samples after an overnight fast were collected in EDTA tubes; to obtain plasma, they were centrifuged at 1500 *g* for 10 min at room temperature. Plasma samples were stored at -80°C in cryovials until further processing.

Sample Preparation and Two-Dimensional Electrophoresis

Samples were prepared as previously described [19–21]. Briefly, 10 μl of plasma (0.5 mg of protein) was mixed with 20 μl of lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 2% ampholytes, pH 3-10, a protease inhibitor mixture). The protein concentration in the sample was determined using the Bradford method [22]. Proteins were separated by isoelectric focusing (IEF) using ImmobilineDryStrip pH 4–7 and 5–8, 7 cm and 13 cm (GE Healthcare) and following the manufacturer's protocol. Samples in lysis buffer were mixed with rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.3% DTT, 0.5% IPG buffer, pH 4–7 or 5–8, 0.001% bromophenol blue) to final volume 130 μl (150 μg protein) per 7 cm strip, or 300 μl (500 μg protein) per 13 cm strip. The strips were rehydrated passively at 4°C for 4 h. IEF was carried out on a Hoefer™ IEF100 device (Thermo Fisher Scientific) programmed as follows: the first stage — 300 V, 1 h, the second stage — a gradient of 300–1000 V, 1 h, the third stage — a gradient of 1000–5000 V, 1.5 h, the fourth stage — 5000 V, 1 h, temperature 20°C and maintained at the voltage 500 V. After IEF, the strips were soaked (2 times for 10 min) in the equilibration solution (50 mM Tris-HCl (pH 6.8), 6 M urea, 2% sodium dodecyl sulfate (SDS), 30% glycerol), containing first 1% DTT and then 5% iodoacetamide. The strips were placed on 14% polyacrylamide gel of the second direction, sealed with 1 ml of a hot solution of 0.5% agarose in electrode buffer (25 mM Tris (pH 8.3), 200 mM glycine, 0.1% SDS) and run electrophoresis in the second direction, using the Hoefer miniVE system (gel size 80×90×1 mm, GE Healthcare) or Ettan™ DALTsix (gel size 18×20×1 mm, GE Healthcare). Electrophoresis was carried out at a constant power of 3.5 W per gel and room temperature. One-dimensional electrophoresis was performed under the same conditions with a polyacrylamide concentration of 14% in the separating gel and 5% in the concentrating gel [23, 24]. The gels were stained with Coomassie R350, scanned using ImageScanner III (GE Healthcare) and analyzed using Image Master 2D Platinum 7.0 (GE Healthcare) and SameSpot (TotalLab, UK) software. The resulting protein maps were then used to determine the coordinates of individual spots and the proteins located in the spots.

Immunoblotting (Western Blot)

Proteins from the gel were transferred to a PVDF membrane (Hybond-P, 0.2 μm, GE Healthcare) in a semi-dry manner for 2 h at 28 V, placing the gel and membrane between two sheets of thick transfer paper (Bio-Rad, soaked in buffer (48 mM Tris, 39 mM glycine, 0.037% SDS, 20% ethanol). After transfer, the membrane was processed according to the Blue Dry Western protocol [25]: stained with 0.1% Coomassie R350, dried and treated with antibodies. Mouse monoclonal anti-Hp (C8, sc- or F8, sc-390962 from Santa Cruz Biotechnology, USA) were used as primary antibodies at a dilution of 1/25 (80 ng/ml) in TBS (25 mM Tris (pH 7.5) and 150 mM NaCl) containing 3% BSA). Secondary horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (NA931V, GE Healthcare) was used in TBS containing 3% defatted dry milk (1/5000 dilution). The reaction was developed using ECL (Western Lightning Ultra, PerkinElmer, USA) and X-ray film (Amersham Hyper Film ECL, UK) with exposure from 10 s to 30 min.

Mass Spectrometry

All procedures were performed following the previously described protocol [23, 26]. After separation with 2DE and staining with Coomassie R350, gel pieces (about 1.5 mm in diameter) corresponding to protein spots were excised using micropipette tips and partially destained by 15 min incubation in 500 μl 50% acetonitrile (ACN) containing

25 mM ammonium bicarbonate (ABA) [23]. Next, the pieces were incubated for 10 min in 200 μl of 100% CAN, which was then removed and the gel was dried for at least 20 min in a Speed Vac centrifuge (Thermo Fisher Scientific). The dried gel pieces were soaked for 25 min on ice in 12 μl of 25 mM ABA solution containing trypsin (Trypsin Gold, 10 μg/ml). For hydrolysis, the original trypsin solution (0.1 mg/ml) was diluted 1:10 with 25 mM ABA, and 100 μl of diluted trypsin was added to each tube. Samples were incubated at 37°C for 4–24 h. Peptides were extracted by adding 150 μl of 60% ACN with 0.1% trifluoroacetic acid (TFA) to each tube containing gel pieces. The extracts were dried in a Speed Vac centrifuge, dissolved in 20 μl of 0.1% TFA, and analyzed using an Orbitrap Q-Exactive Plus mass spectrometer (Thermo Fisher Scientific). Protein identification and relative quantification were performed using Mascot 2.4.1 (Matrix Science) and emPAI (exponentially modified form of protein abundance index) [23].

RESULTS AND DISCUSSION

In order to analyze the Hp proteoforms in more detail and assess the extent of their variability, we have performed a more detailed analysis of plasma samples using mass spectrometry and antibodies to Hp. In the case of enzyme immunoassay, proteins after 2DE separation were transferred from the gel to the membrane by electrotransfer and analyzed (Western blotting) using antibodies to the α- or β-chain of Hp. It should be noted that enzyme immunoassay using antibodies to the α-chain of Hp detects not only proteoforms of the α-chain itself (region 16–18 kDa), but also the full-length, unprocessed version of Hp, known as zonulin, which has a mass of ~45 kDa [27]. The 45 kDa polypeptide that interacts with antibodies to the α chain may be identified as zonulin. This polypeptide was found both in a standard position, corresponding to the theoretical parameters of zonulin (p*I* 6.13 / Mw 45000), and in an unusual position with the isoelectric point shifted to the alkaline region (pI ~8) [16]. This confirms our previous mass spectrometric data obtained by sectional analysis about the presence of this form in the plasma of a GBM patient [16]. However, this proteoform was found in only one GBM patient. It was absent in the control group and other patients. Thus, variability is detected at the level of the full-size form (zonulin) of Hp, and the presence of the alkaline form of zonulin in plasma can be considered a conditional, but insufficient, biomarker that may indicate the presence of GBM.

In this work, our main attention has been paid to the Hp β-chain because its variability is determined primarily by post-translational modifications (PTMs). Different α-chain proteoforms can also be generated due to individual phenotype/genotype [11]. For a better comparison of our results and literature data, Figure 1A shows results from the SWISS-2DPAGE database [18]. Separation with better resolution was carried out using 13 cm strips (pH 4–7). In this case, the gel was stained, the spots were cut out, and mass spectrometry analysis of the proteins present in them was carried out. As a result of these combined efforts, a detailed composite map of the distribution of the β-chain proteoforms as well as the full-length form of Hp (zonulin) was obtained after analysis of plasma samples from various donors (Fig. 1B). The image analysis of 2DE gels, where the position of individual proteoforms was determined by means of mass spectrometry and immunodetection, a diagram of the location of β-chain proteoforms/spots was created (Fig. 1B, Table 1). The Hp content of the spots after Coomassie R350 staining was determined using mass spectrometry. Attention should be paid to the important fact that each spot may contain more than one protein. Even the most major Hp spots contain no more than 60% of this protein. At the same time, minor forms of Hp may be present in spots of other proteins: ANT3, A1AT, VTDB, CLUS, FIBG [16]. The presence of other proteins in the spots can explain the fact that in some samples identically positioned spots contain Hp, but others lack it.

The distribution of Hp β-chain protein spots shows that while the most major spots were well reproducible in different samples, the minor spots in this region varied greatly. There are indications in the literature that the distribution of at least major spots is determined by the degree of their glycosylation [28]. For a more detailed analysis of minor spots of the Hp β-chain, 2DE was performed using two types of strips (pH range 5–8, length 7 cm and pH range 4–7, length 13 cm). The second strips were also used for immunoblotting. A total of 9 plasma samples from healthy donors, 7 from GBM patients and 1 plasma sample from a patient with cervical cancer were analyzed. Since treatment of the entire surface of the membrane with antibodies provided preferential signal from the major spots of the Hp β-chain masking the signals from adjacent spots (Fig. 1C), their local targeted treatment was used. For this purpose we have used the “Blue Dry Western” immunodetection method as PVDF membrane pre-staining allows to treat specifically certain its areas and the hydrophobicity of the dried membrane prevents solutions from spreading over the entire surface. Figures 2–4 show examples of such analysis.

A total of 49 different proteoforms (spots) of the Hp β-chain were detected. Eight of them are major ones present in all plasma samples. The ratio of the level of their presence (the intensity of the corresponding spots) is not the same in different samples. Other (minor) proteoforms were present in some (but not all) plasma samples. Their combination in each sample was different, and in each individual case there were no more than 33 proteoforms out of 49 possible (Table 1). The main conclusion from the results is that there was no correlation

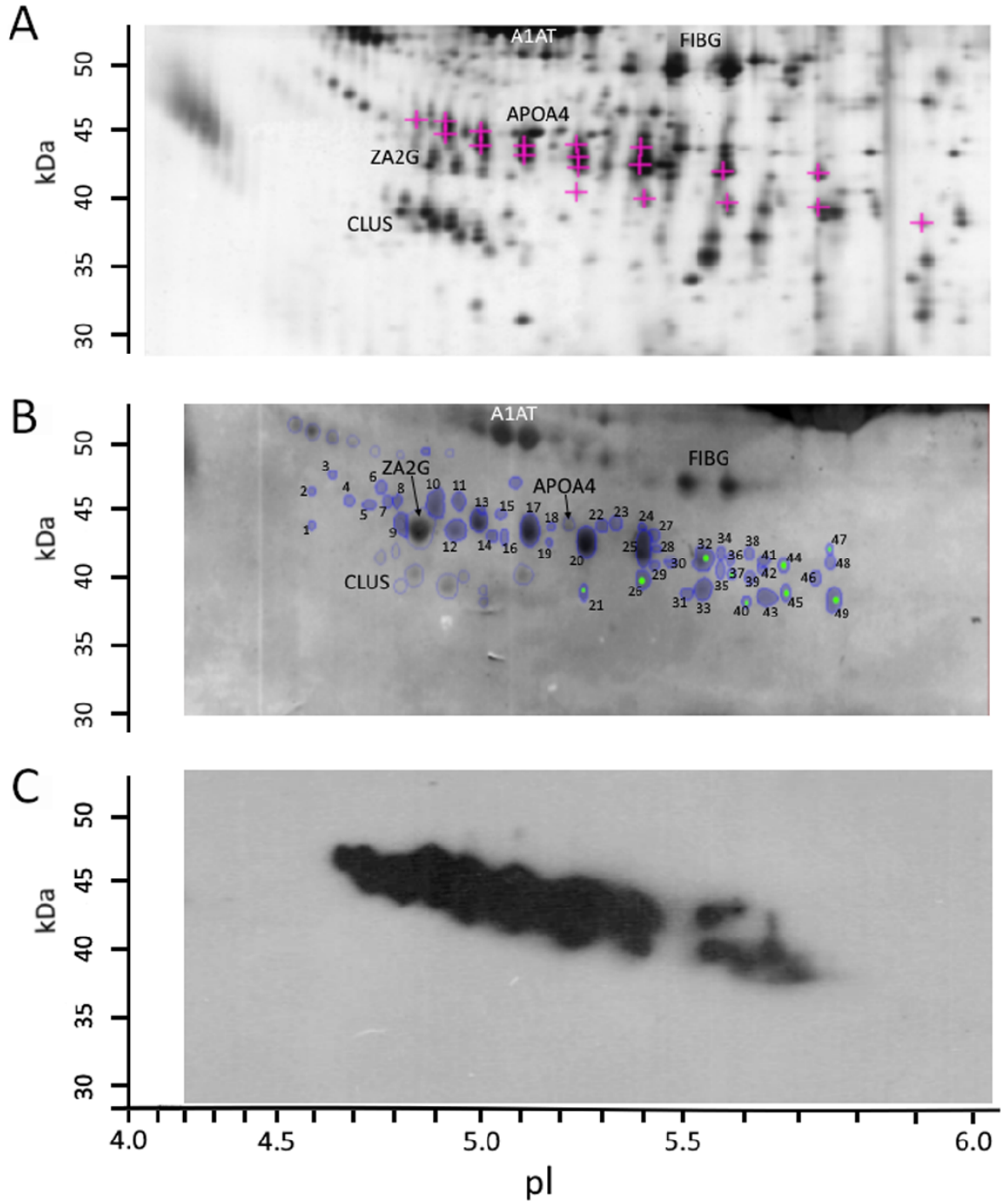

Figure 1. Schematic position of the Hp β-chain spot. **A** – the enlarged area of β-chain spots on a two-dimensional map of human plasma proteins from the SWISS-2DPAGE database [18]. **B** – the composite map of the distribution of Hp β-chain proteoforms, constructed on the basis of 2DE samples with subsequent immunological and mass spectrometric detection. **C** – immunodetection of the β-chain after 2DE of plasma sample no. 15.

associated with the presence of any of these proteoforms only in samples from cancer patients. In other words, we can state that there is only individual variability in the proteoforms of the Hp β-chain, regardless of the health state of the donor.

In a separate series of experiments, membranes were also treated with antibodies to the α-chain. In this case, plasma samples from 11 donors were analyzed (6 controls, 4 GBM patients, 1 cervical cancer patient). The signal for antibodies to the α-chain, localized in regions of molecular weight 45 kDa, was detected in approximately half of the plasma samples from oncology patients (2 — GBM, 1 patient with cervical cancer). Zonulin was not detected in control samples and some cancer samples (8 out of 11 samples).

Table 1. Presence of detected proteoforms (spots) of the Hp β-chain in the analyzed plasma samples. The α symbol indicates the position of zonulin detected by antibodies to the Hp α-chain. CC – cervical cancer

2DE	pH 4–7, 13 cm										pH 5–8, 7 cm								
	Control						CC	Glioma			Control			Glioma					
Spot/ sample no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	8	9	15	16	17
1								+											
2	+	+			+				+										
3	+				+	+	+			+									
4	+			+	+	+	+	+	+	+									
5				+	+		+												
6	+	+			+				+	+									
7	+	+			+				+										
8	+	+		+		+	+	+											
9	+	+	+		+	+	+	+	+	+									
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+		+		+	+		+	+						+			
12	+	+	+	+	+	+	+	+	+	+					+	+			
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14			+	+		+		+	+	+					+				
15	+	+	+	+	+		+		+							+			
16	+		+	+			+												
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	+		+	+	+		+												
19	+						+		+							+			
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+		+				α	+	+		+	+	+	+	+	+	+	+	+
22			+				+		+		+	+	+	+		+	+	+	+
23	+	+	+		+		+	+	+						+	+	+	+	+
24		+						+	+		+	+	+	+	+	+		+	+
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
26	+	+	+	+	+	+	+	α	+	+	+	+	+	+	α	+	+	+	+
27	+			+	+		+		+										
28					+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29											+								+
30				+				α			+			+	α				
31	+	+		+			+		+		+					+			
32	+	+	+	+	+	+	+	α	+	+	+	+	+	+	α	+	+	+	+
33	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
34	+		+		+														
35		+			+														
36		+					+		+	+						+			
37							+	α	+	+					α	+			
38	+	+	+					+	+		+	+	+	+	+	+	+	+	+
39									+		+		+	+		+	+	+	+
40	+						α		+		+					+			
41	+		+	+	+	+			+	+		+	+	+		+	+	+	+
42			+																
43	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
44		+						α							α				
45	+	+			+				+	+	+					+			
46							+		+							+		+	+
47			+				α												
48			+				+				+		+	+			+	+	
49	+	+	+		+	+	α		+	+	+	+	+	+		+	+	+	+
Total spots	32	26	25	22	27	18	28	18	34	22	22	16	18	19	14	28	18	20	20

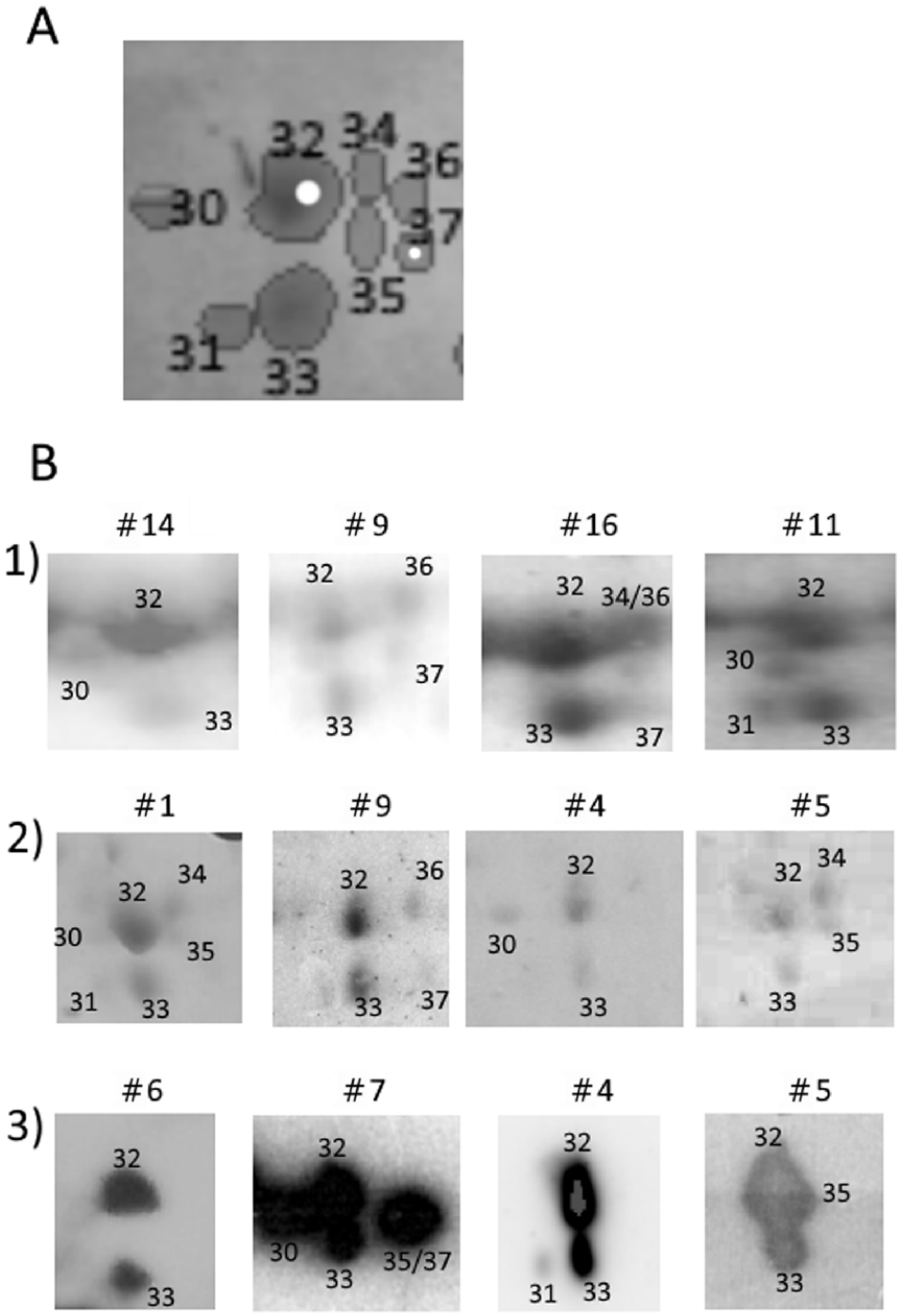

Figure 2. Identification of the Hp β-chain proteoforms in the cluster of spots 31-37. **A** – composite scheme of spots, **B** – gels and immunoblots: *1*) 2DE gels, Coomassie R350 staining, pH 5-8, 7 cm, *2*) 2DE gels, Coomassie R350 staining, pH 4-7, 13 cm, *3*) 2DE immunoblots, pH 4-7, 13 cm.

Since PTMs and their various combinations are the main source of proteoforms, we must take into consideration that the Hp β-chain can be N-glycosylated (4 sites), acetylated (4 sites), phosphorylated (5 sites), ubiquitinated (1 site) (Phosphositeplus, [29]). It is easy to find that a combinatorial count, using formula (1), shows in this case the possibility of the existence of at least 4000 different proteoforms of the Hp β-chain:

$$C_n^k = \frac{n!}{k! \times (n-k)!} \quad (1),$$

where n is the number of objects (in this case, this is the number of all PTMs), k is the number of combinations of objects (in this case, the number of different PTMs from 0 to 12).

CONCLUSIONS

The results obtained in this study were quite unexpected. If the total level of plasma Hp in cancer patients, especially patients with GBM, was increased

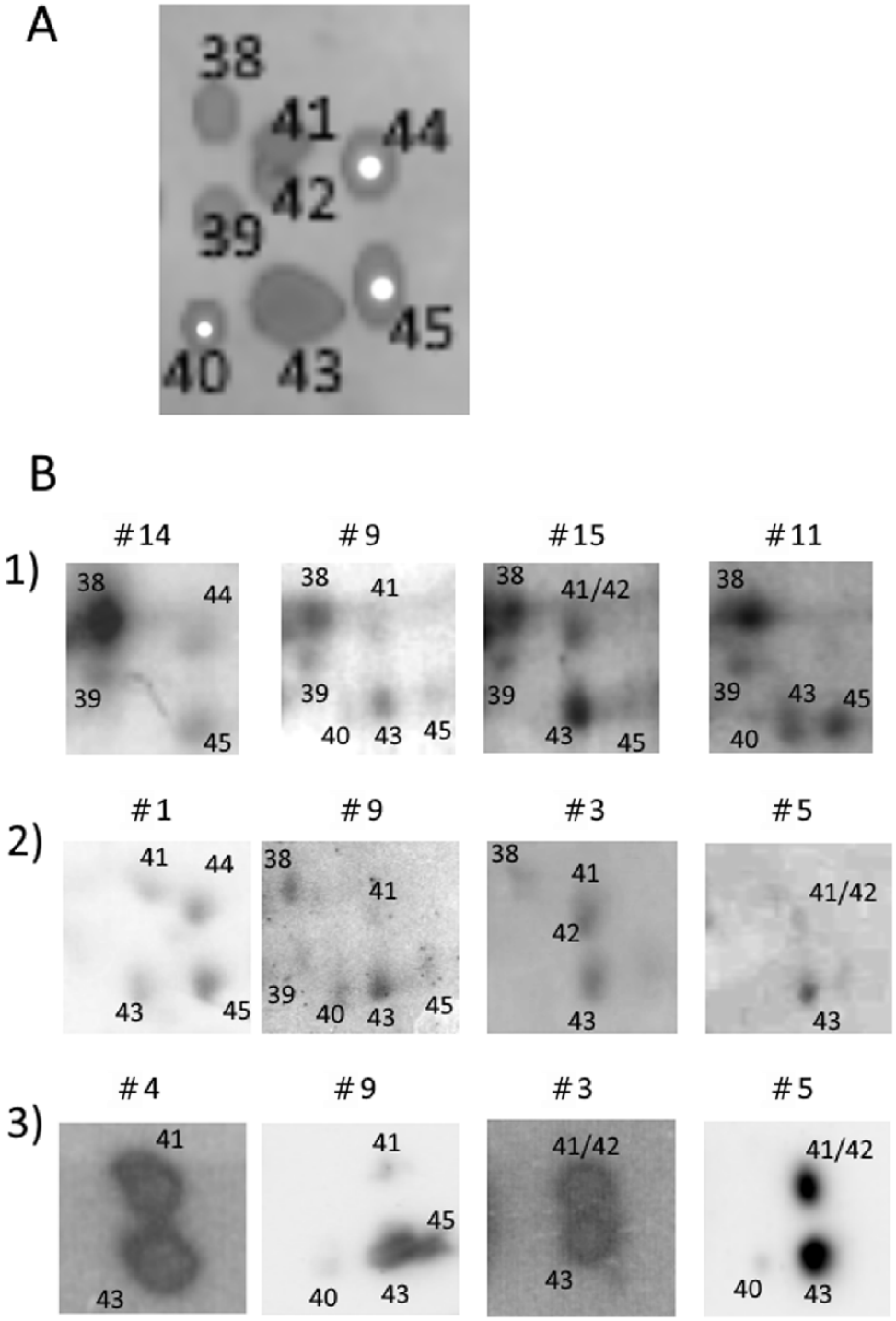

Figure 3. Identification of the Hp β-chain proteoforms in the cluster of spots 38-45. **A** – composite scheme of spots, **B** – gels and immunoblots: *1*) 2DE gels, Coomassie R350 staining, pH 5-8, 7 cm, *2*) 2DE gels, Coomassie R350 staining, pH 4-7, 13 cm, *3*) 2DE immunoblots, pH 4-7, 13 cm.

compared to the norm, then the presence of individual proteoforms, detected by their position on the two-dimensional map, was very individual. In our case, from 16 to 33 different proteoforms of the Hp β-chain were detected in each individual plasma sample, and a total of 49 were detected in all analyzed samples. However, taking into consideration the relatively small sample set used in this experiment, it is likely that a much larger number of possible Hp β-chain proteoforms could be found if more plasma samples from other donors are analyzed. A very important conclusion from the results obtained can be drawn if we assume that the detected variability in the Hp β-chain is present to varying degrees in other proteins. In this case we can assume that the main contradiction that is found during calculation of the number of possible and actually detectable human proteoforms is most likely associated

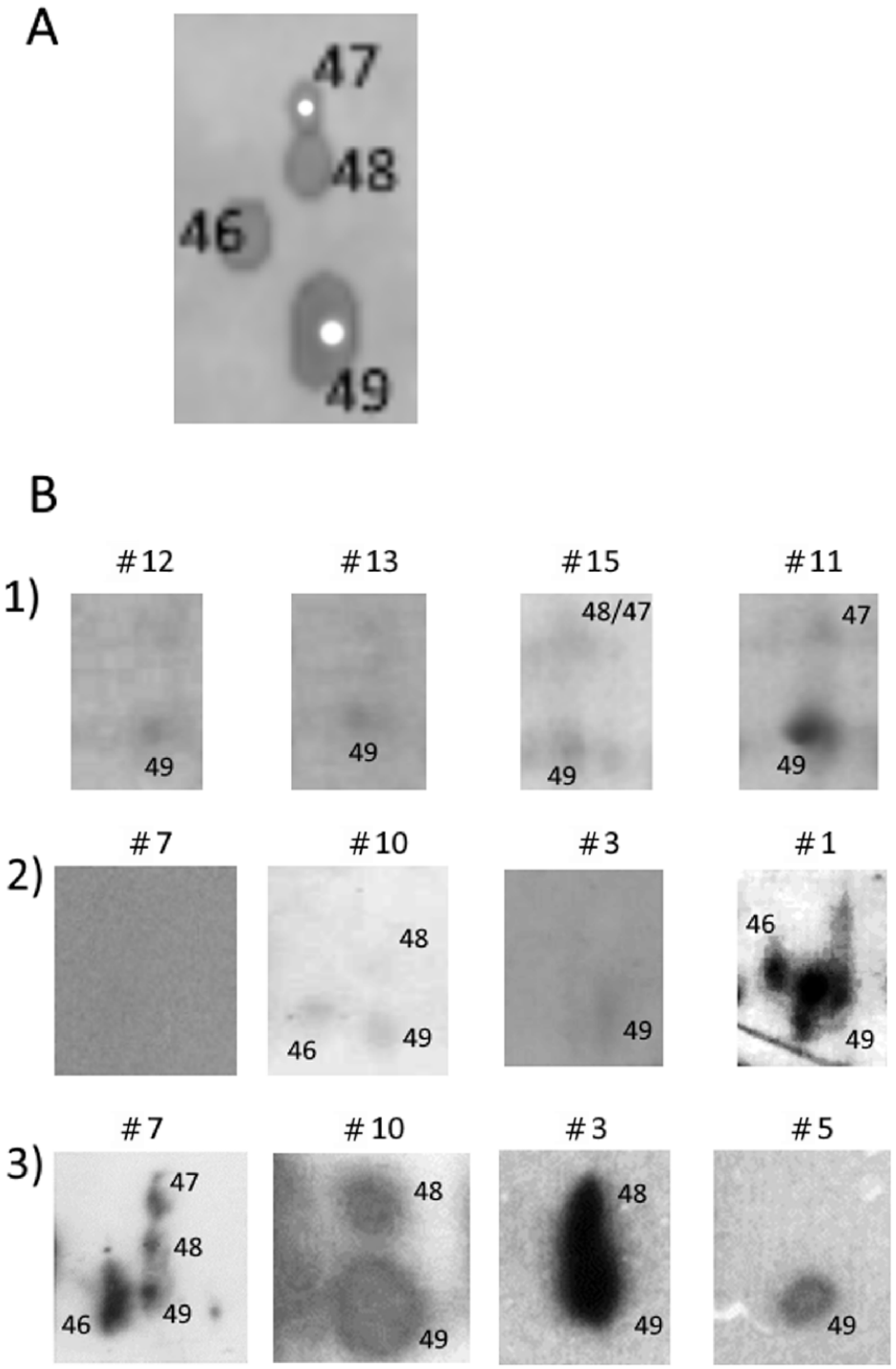

Figure 4. Identification of the Hp β-chain proteoforms in the cluster of spots 46-49. **A** – composite scheme of spots, **B** – gels and immunoblots: *1*) 2DE gels, Coomassie R350 staining, pH 5-8, 7 cm, *2*) 2DE gels, Coomassie R350 staining, pH 4-7, 13 cm, *3*) 2DE immunoblots, pH 4-7, 13 cm.

with individual variability [30–33]. Moreover, among the total possible number of options (proteoforms), only a limited part is realized in each individual case. Their formation occurs in such a way that the main, most major, proteoforms are present in all individuals, but sets of many minor forms arise in different individual ways.We may suggest that, on the one hand, these proteoforms are a product of stochastic noise and do not have a special effect on the functionality of the protein molecule, and on the other hand, such a wide variety of proteoforms can serve as an evolutionary mechanism. Although all these speculations certainly require additional experimental validation, the presence of such strong Hp variability at the level of β-chain proteoforms (along with different α-chain phenotypes) may have practical significance and serve as an analogue of fingerprints at the molecular level.

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COMPLIANCE WITH ETHICAL STANDARDS

The study was conducted in accordance with the recommendations of the Declaration of Helsinki and was approved by the Local Ethical Commission of the St. Petersburg Institute of Nuclear Physics of the National Research Center "Kurchatov Institute" (protocol code 02_2020 dated April 21, 2020). All patients signed informed consent forms for participation in the study and provision of biological material for study.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ВАРИАБЕЛЬНОСТЬ ПРОТЕОФОРМ БЕТА-ЦЕПИ ГАПТОГЛОБИНА

Н.Л. Ронжина[1], Е.С. Зорина[2], М.Г. Завьялова[2], О.К. Легина[1], С.Н. Нарыжный[1]*

[1]Петербургский институт ядерной физики им. Б.П. Константинова
Национального исследовательского центра “Курчатовский институт”,
188300, Ленинградская область, Гатчина, Орлова роща, 1; * эл. почта: snaryzhny@mail.ru
[2]Научно-исследовательский институт биомедицинской химии им. В.Н. Ореховича,
119121, Москва, Погодинская 10

Имеющаяся обширная информация об изменениях, которым подвергается молекула гаптоглобина (Hp), свидетельствует о возможности его существования в виде множества протеоформ, которым, вероятно, свойственны разные функции. С помощью двумерного электрофореза (2DE) в комбинации с масс-спектрометрическим и иммунным детектированием нами были проанализированы образцы плазмы крови как от здоровых доноров, так и от пациентов, у которых была диагностирована первичная глиобластома IV степени (ГБМ), и получена детальная композитная 2DE-карта распределения протеоформ β-цепи, а также полноразмерной формы Hp (зонулина). Оказалось, что если общий уровень Hp в плазме онкобольных, особенно пациентов с ГБМ, повышен по сравнению с нормой, то присутствие отдельных протеоформ, детектированных на основании их положения на 2DE-карте, очень индивидуально. Была обнаружена вариабельность как у зонулина, так и у β-цепи Hp. А присутствие щелочной формы зонулина в плазме может считаться условным, но недостаточным, биомаркером глиобластомы. То есть, мы обнаружили, что на уровне минорных протеоформ Hp даже в норме присутствует высокая индивидуальная вариабельность. С одной стороны, это вызывает вопросы о причинах такой вариабельности, если она присутствует не только у Hp, но и у других белков. С другой стороны, это может объяснить несовпадение количества экспериментально детектируемых протеоформ с теоретически возможными не только у Hp, но и у других белков.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: гаптоглобин; протеоформы; глиобластома; индивидуальная вариабельность

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