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Characterization of novel peptide-specific antibodies against the translation elongation factor eEF1A2 and their application for cancer research

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Aim. We intend to characterize the new peptide-specific antibodies against the isoform 2 of translation elongation factor 1A (eEF1A2) and determine its presence in the postoperative samples of human breast, lung and stomach tumor tissues. **Methods.** The analysis of antibody specificity was performed by enzyme-linked immunosorbent assay, immunoblotting and immunohistochemistry. Immunoblotting and immunohistochemistry were used for the determination of the eEF1A2 in the human tumor samples, as well as in the samples of normal tissues surrounding tumors. **Results.** The antibodies obtained against the eEF1A2 specifically recognized this protein in the cell extracts and histological sections and did not cross-react with the elongation factor 1A isoform 1. eEF1A2 was revealed in the postoperative samples of breast, lung and stomach tumors as well as in the putative normal tissues surrounding tumors. **Conclusions.** The antibodies obtained against eEF1A2 are highly specific for the antigen and can be used for the immunological studies of tumors.

Keywords: eEF1A2, anti-peptide antibodies, immunoblotting, immunohistochemistry.

Introduction. Eukaryotic translation elongation factor 1A (eEF1A) provides the process of peptide chain elongation in a cell via delivering the aminoacylated tRNA to the A-site of the ribosome [1]. Moreover, eEF1A is also involved in the functioning of other cellular processes not directly related to the translation [2–5].

In vertebrates, unlike other organisms, two isoforms of translation elongation factor 1A, named eEF1A1 and eEF1A2, are present. Despite the fact that these isoforms are encoded by different genes, they share 92 % of iden-

tity of their amino acid sequences [6]. Interestingly, the expression of both isoforms in an organism is mutually exclusive. The presence of eEF1A2 is usually limited to neurons, muscle cells and cardiomyocytes. However, during carcinogenesis this isoform appears in those tissues, where it is absent in the normal state [7–9]; this fact may point to the eEF1A2 oncogenic properties [7, 8]. Until now the reason of mutually exclusive distribution of eEF1A isoforms in an organism and the mechanism of induction of eEF1A2 expression in tumors remain unknown. The appearance of eEF1A2 in some tissues after their malignant transformations suggests the

existence of a specific signaling pathway, which may «turn on» the expression of certain set of genes including *EEF1A2*. Recently, a considerable evidence for the microRNA-mediated regulation of the *EEF1A2* expression has been obtained [10].

Since eEF1A1 and eEF1A2 isoforms differ in physical and chemical properties [11, 12] and differently interact with some signaling proteins [13], the non-canonical functions of these isoforms in a cell, particularly those associated with carcinogenesis, may also vary. For example, certain studies demonstrated the anti-apoptotic properties of eEF1A2, whereas eEF1A1, by contrast, was considered to be the pro-apoptotic protein [14, 15], although some authors also observed the anti-apoptotic effect of the latter [16, 17].

The appearance of eEF1A2 in the samples of the breast [8, 18], pancreas [19], ovarian [20–22], lung [9, 23], liver [24] and stomach [25] cancer suggests a role of this protein in tumorigenesis, but its exact function in cancer cells remains unknown. It should be noted that the eEF1A2 expression in tumors was determined in most cases either at the mRNA level or with the help of commercial antibodies, which were not checked for the absence of cross-reactivity with eEF1A1. Only one laboratory in the world reported the use of eEF1A2-specific antibodies for the location of this eEF1A isoform in tissues [8, 20]. At present, this antibody preparation is not commercially available.

In order to study the eEF1A2 function in tumorigenesis one must have a tool to detect this protein in the cells and cell extracts. Currently, a monospecific antibody preparation is the only appropriate tool that can be made. Recently, we have shown that mouse immunization with one of the eEF1A2 fragments conjugated to bovine serum albumin (BSA) led to the generation of antibodies that specifically interacted with purified eEF1A2, but not with eEF1A1 [26]. In this paper we characterize the peptide-specific antibodies obtained after immunization of goat with the abovementioned peptide conjugated to hemocyanin and ovalbumin. The specificity of the antibody preparation was tested with both purified isoforms of translation elongation factor 1A as well as at the histological sections and cell extracts that contained only one isoform. Using this antibodies, we also detected eEF1A2 in the human postoperative malignant tissue samples of several organs.

Materials and methods. Synthesis of antigenic peptide KSDPPQEAAQFTSQ, its purification and conjugation to keyhole limpet hemocyanin and ovalbumin were performed as described in [26]. Conjugates were characterized by determining the number of peptide copies per molecule of conjugate by MALDI-TOF mass spectrometry [26].

Immunization of goat was performed as follows. The first injection (day 0) was made with 1 mg peptide-hemocyanin conjugate in 2 ml of 1:1 (v/v) mixture of Complete Freund's Adjuvant (CFA) and 0.9 % NaCl water solution. The second and third injections (14th and 28th days) were made with 1 mg peptide-hemocyanin conjugate in 2 ml of 1:1 (v/v) mixture of Incomplete Freund's Adjuvant (IFA) and 0.9 % NaCl water solution. All injections were made subcutaneously (s. c.). The first test-bleeding (20 ml) was done on 35th day, and the serum antibody titer was determined by ELISA using native and denatured eEF1A2 protein. The fourth and fifth s. c. injections (42th and 56th days) were done with 1 mg peptide-ovalbumin conjugate in 2 ml of 1:1 (v/v) mixture of CFA and 0.9 % NaCl water solution. One week after the fifth injection the preparative bleeding (100 ml) was performed and the serum was obtained. IgGs were further purified by ammonium sulfate precipitation (40 % saturation) and after dialysis on Blue DEAE-Affi-gel column according to the manufacturer's recommendations («Bio-Rad Laboratories», USA). Antibodies were finally purified on an affinity column with immobilized peptide. The affinity matrix was made by ligating the antigenic peptide to N-hydroxysulfosuccinimide-activated Affi-gel 15 resin according to the manufacturer's recommendations («Bio-Rad Laboratories»). 4 mmoles of peptide were immobilized onto 1 ml of Affi-gel matrix. The IgG fraction obtained after Blue DEAE-Affi-gel was loaded directly on an affinity column, which was pre-equilibrated with a sample buffer containing 20 mM Tris-HCl, pH 8.0, 25 mM NaCl and 0.02 % (w/v) NaN₃. The column was extensively washed by the same sample buffer to remove nonspecifically adsorbed proteins. The peptide-specific antibodies were eluted from the column by 0.1 M glycine-HCl, pH 2.4 buffer containing 0.025 % NaN₃. The eluate fractions (7 ml) were collected into tubes with 467 µl of 1 M Tris-HCl buffer, pH 8.5 that were periodically stirred to reach a neutral pH value. Then the fractions were

concentrated using Vivaspin 6 PES concentrators (30 kDa pore size, «Sartorius», Germany) to 0.5 ml. After that, the antibody fractions were diluted by 5 ml PBS, followed by concentrating in the same concentrator tube. This procedure was repeated three times in order to replace the previous sample buffer solution with PBS. After the last round of concentration the antibody titers and protein concentrations were measured as described in [26]. The fractions with the highest titer were diluted with glycerol up to 50 % (v/v) and stored at -20°C .

Individual eEF1A1 and eEF1A2 proteins from rabbit tissues were purified as described previously [12, 27].

Samples of normal rabbit tissues, including muscle, heart (tissues containing eEF1A2), liver and lung (tissues containing eEF1A1) and postoperative tissue samples of lung, kidney, breast, stomach malignant tumors as well as respective normal tissues surrounding tumors were employed in immunohistochemical studies of eEF1A2 localization. The samples of normal tissues were taken after euthanasia of an animal.

Paraffin blocks of postoperative samples of human malignant tumors, control tissues and normal rabbit tissues were prepared according to a standard histological technique.

Paraffin blocks were sliced by the microtome («Microm» HM430, «Karl Zeiss», Germany). Immunohistochemical study of tissue samples was carried out according to the protocol described previously [28]; the mandatory control staining of tissue sections was performed via using only secondary antibodies («Control»). Working dilution of the anti-peptide antibody preparation against eEF1A2 is specified in the figure legends. Pierce[®] Mouse Anti-Goat IgG («Thermo scientific») conjugated to biotin was used as a secondary antibody with the working dilution 1:250. Ultra-Sensitive ABC Peroxidase Staining Kit («Thermo scientific») was used for the visualization of immune complexes with anti-peptide antibodies.

Commercially available Anti-EF1 α antibody preparation (clone CBP-KK1, «Upstate», USA) with the working dilution 1:100 was used for the detection of both eEF1A isoforms. The immune complexes were visualized with the help of «UltraVision Quanto Detection System HRP» («Thermo scientific»). Photodocumentation of the immunohistochemistry results was done on «Leica DM1000» microscope (Germany) using 40 \times objective.

Immunoblotting of proteins was performed as described in [28]. The secondary antibodies (1:5000 dilution) against goat IgGs conjugated to horseradish peroxidase («Imtek», Russian Federation) were used for detection of primary anti-peptide antibodies. Peroxidase-conjugated rabbit antibodies against mouse IgGs («Sigma», USA) were used at 1:10,000 dilution in immunoblotting with Anti-EF1 α (clone CBP-KK1, «Upstate», USA). Visualization of immune complexes on the blotting membrane was done using Immobilon Western Chemiluminescent HRP substrate from «Millipore» (USA). Immunoblotting images were obtained on «ChemiDoc» («Bio-Rad Laboratories», USA).

Results and discussion. *Characterization of anti-peptide antibodies against eEF1A2 by immunohistochemistry and immunoblotting.* Recently, we have shown that the immunization of mice with KSDPPQEAAQFT SQ peptide conjugated to BSA led to the generation of antibodies that specifically recognized eEF1A2, but not eEF1A1 [26]. However, mouse immunization did not allow obtaining the amount of antibodies sufficient for multiple histochemical trials; hence, a large-scale anti-peptide antibody preparation via goat immunization was started. The same eEF1A2 fragment was used as a peptide antigen, which was conjugated to hemocyanin (3–6 peptide copies per the hemocyanin monomer conjugate) and to ovalbumin (2–5 peptide copies per the conjugate molecule). The peptide conjugated to hemocyanin led to the generation of antibodies (Supplementary, Fig. S1) that recognized the native eEF1A2 (titer 1:2000) better than the denatured one (titer 1:1000). Further immunization with the peptide-ovalbumin conjugate resulted in an increase of the antibodies titer against denatured eEF1A2 up to 1:2000 (Supplementary, Fig. S2), thus making the antibody preparation more suitable for immunoblotting and immunohistochemical experiments. After the affinity purification, 1 μg of affinity-purified peptide-specific IgG was enough to detect 0.05 μg eEF1A2 in ELISA.

Primarily, the specificity of affinity-purified antibodies against eEF1A2 was tested using individual eEF1A1 and eEF1A2 proteins. As shown in Fig. 1, *A*, the antibodies against eEF1A2 did not cross-react with eEF1A1 even at two fold excess of the latter on the membrane. On the other hand, the commercial antibody against eEF1A recognizes both isoforms (Fig. 1, *B*). We further tested the anti-peptide antibodies using the extracts

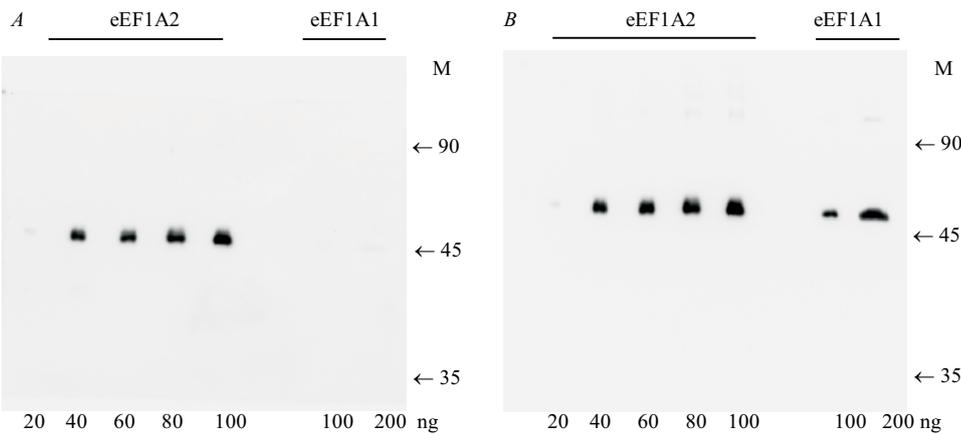


Fig. 1. The anti-peptide antibodies recognize the individual eEF1A2, but not eEF1A1, in Western blot: *A* – the membrane was treated with the primary anti-peptide antibodies (0.24 µg/µl) against eEF1A2; *B* – the membrane was treated with commercial antibody that recognizes both isoforms of eEF1A. M – prestained molecular weight proteins markers, kDa («Thermo scientific»)

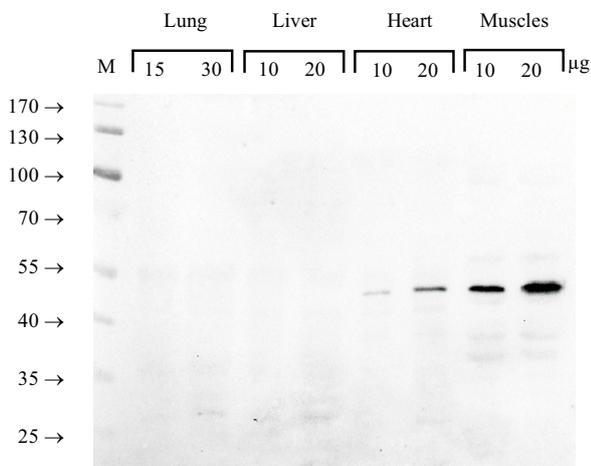


Fig. 2. The anti-peptide antibodies recognize eEF1A2 protein in the heart and muscles extracts in the Western blot. Working dilution of the antibodies was 0.24 µg/ml. Time of membrane exposition was 1 min; M – molecular weight proteins markers, kDa (PageRuler «Thermo scientific»)

of normal rabbit tissues that contained different isoforms of translation elongation factor: muscle and heart, as eEF1A2 positive tissues, and liver and lung, as eEF1A2 negative tissues. Rabbit tissues were chosen because both isoforms of eEF1A is 100 % identical to the corresponding human proteins. As shown in Fig. 2, anti-eEF1A2 antibodies recognized 50 kDa band in the extracts of rabbit muscle and heart, but no signal was observed in the extracts of liver and lung. Thus, the immunoblotting analysis of tissue extracts confirmed a high specificity of the prepared antibodies and validated their use for the immunohistochemical studies.

Anti-peptide antibodies against eEF1A2 were employed for the immunohistochemical detection of eEF1A2 in sections of rabbit muscles, heart, lung and liver (Fig. 3). The sections of rabbit heart and muscles demonstrated

a strong positive reactivity (intense brown) for eEF1A2. To the contrary, the lung and liver tissue sections were not stained after the treatment with the same antibodies. The control sample treatment with the secondary antibodies only resulted in no staining. When the commercial antibody that recognizes both isoforms of eEF1A was used, all tissues were positively stained (Supplementary, Fig. S3). Thus, the anti-peptide antibodies against eEF1A2 specifically recognized this protein at tissue sections containing eEF1A2, but not eEF1A1 that confirmed their suitability for immunohistochemical studies.

Determination of eEF1A2 in human tumor samples by immunohistochemistry and immunoblotting. Paraffin sections of postoperative material were employed for the determination of eEF1A2 in the samples of human lung and breast tumors by immunohistochemical analysis with the help of anti-eEF1A2 anti-peptide antibodies. The tumor samples were compared to those of the normal tissue that surrounded the tumor and considered as healthy.

Since eEF1A2 protein was previously detected in breast tumors by immunohistochemistry using the antibodies from another laboratory [8, 18], we decided to verify this result using our peptide-specific antibodies. We examined 4 pairs (tumor and normal tissues) of postoperative samples of the human breast cancer (Fig. 4). The clusters of big cells with brown cytoplasm and nuclei were observed in the tumor samples (BrCr#). The morphology of these cells was atypical compared to the normal lung cells; hence they could be considered as the cancerous ones. Unexpectedly, the putative normal samples (BrN#) were also stained brown though less inten-

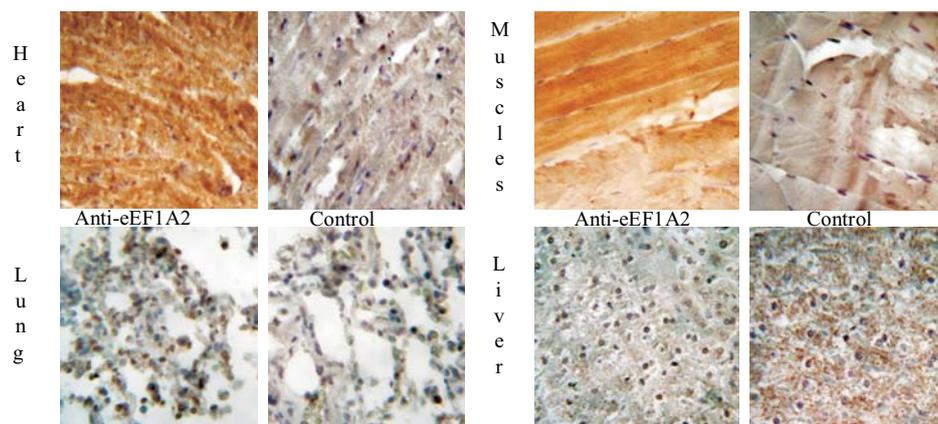


Fig. 3. Immunohistochemical staining of the normal rabbit tissue sections with the peptide-specific antibodies against eEF1A2. Control samples were treated with the secondary antibodies only. Magnification 40x. Nuclei were counterstained with hematoxylin

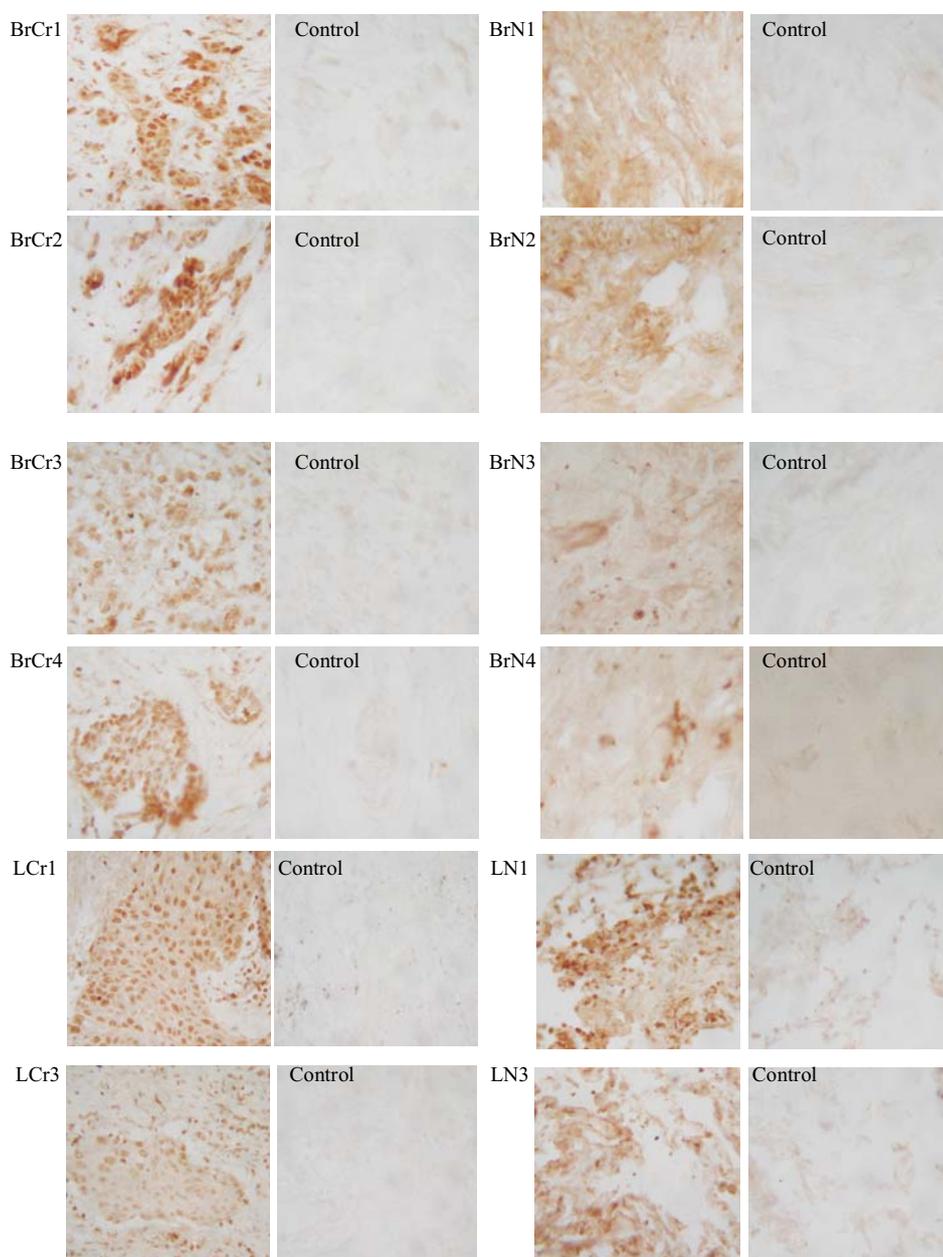


Fig. 4. Immunohistochemical staining of the human breast tumor (BrCr#) and normal (BrN#) tissue sections from different patients with the antibodies specific to eEF1A2. Working concentration of antibodies was 1.2 μg/ml. Control samples were treated with the secondary antibodies only. Magnification 40x

Fig. 5. Immunohistochemical staining of the human lung tumor (LCr#) and normal (LN#) tissue sections from different patients with the antibodies specific to eEF1A2. Working concentration of antibodies was 1.2 μg/ml. Control samples were treated with the secondary antibodies only. Magnification 40x

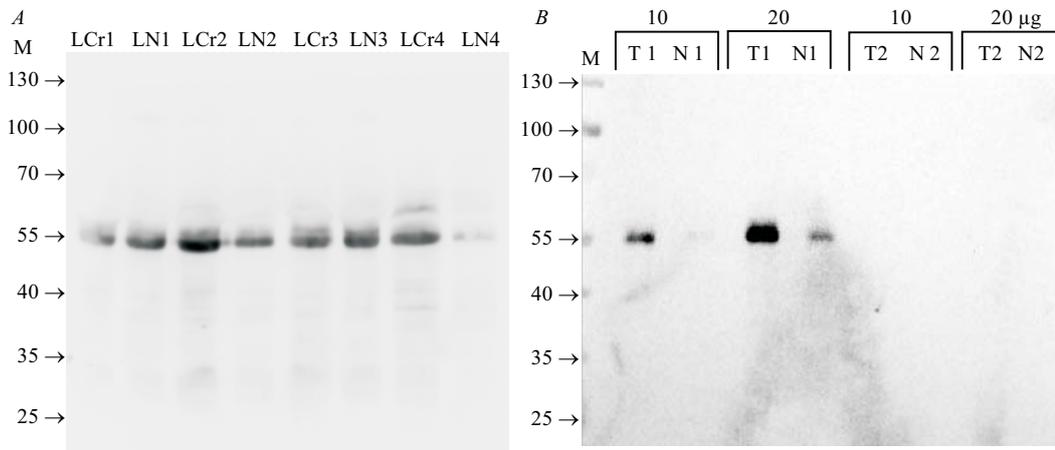


Fig. 6. *A* – Western blot analysis of the human lung cancer (LCr#) and normal tissue (LN#) extracts; *B* – human gastric cancer (T#) and normal tissue (N#) extracts with the peptide-specific antibodies against eEF1A2. The working concentration of the antibodies was 0.24 µg/ml. Exposure time was 1 min; M – molecular weight proteins markers, kDa (PageRuller «Thermo scientific»)

sively, whereas the morphology of these tissue samples differed from the tumorous ones.

However, a small number of cells with the atypical morphology and intense staining could also be seen in the samples BrN3 and BrN4.

The same immunohistochemical analyses were performed for the samples (tumor and normal tissues) of human lung cancer (LCr#). The positive staining was observed in all lung tumor samples in the presence of the specific antibodies against eEF1A2 (Fig. 5). Clusters of big cancer cells with brown cytoplasm and nuclei were clearly seen. Normal tissues surrounding the tumors (LN#) were also stained brownish despite the cell morphology in these samples was distinctly different from that in tumor tissues. Consequently, the samples designated as «breast or lung normal» contained eEF1A2 isoform that was detected by the peptide-specific antibodies.

We showed that the anti-peptide antibodies against eEF1A2 specifically recognized this protein in the crude tissue extracts (Fig. 2). This allowed us to screen tumor extracts (postoperative samples) for the presence of eEF1A2 by immunoblotting. A strong signal that corresponds to 50 kDa protein was detected in all extracts derived from tumors and in most extracts, except LN4, of lung normal tissue (Fig. 6, *A*). This result confirms the immunohistochemical data obtained on samples of human lung tumor (Fig. 5). It is worth mentioning that in normal rabbit lung tissue eEF1A2 was not detected (Fig. 2).

Thus, the results of immunohistochemical analysis and immunoblotting confirm the expression of eEF1A2 isoform in certain malignant tumors. It is in accordance with the previous results obtained for the lung cancer samples [9, 23].

We also analyzed two pairs (tumor and normal tissue) of protein extracts derived from patients with gastric cancer (Fig. 6, *B*). Although the signal from eEF1A2 was observed only in one pair of extracts, this protein was still present in both tumor and normal samples.

Hence, we confirmed the expression of eEF1A2 in the lung, breast and stomach malignant tumors and respective normal tissues surrounding the tumors. The presence of eEF1A2 in the normal tissues adjacent to the tumors was somewhat surprising result. Undoubtedly, it requires further studies on a larger number of samples. On the one hand, this fact makes eEF1A2 protein a somewhat compromised tumor marker, on the other hand, it raises the question about the «normality» of cells adjacent to the tumor.

It might be of interest to analyze the correlation between the presence of eEF1A2 in those tissues, where it is absent in the normal state, and the stage of their malignant transformation. Since the anti-peptide antibodies against eEF1A2 do not cross-react with eEF1A1, the positive staining of the normal tissues surrounding the tumors cannot be simply explained by a non-specific effect of the antibodies.

It is known that tumors somehow affect their environment. Cancer cells can influence a surrounding normal tissue directly by paracrine factors or indirectly via stromal cells [29]. It has been shown recently that the fibroblasts extracted from the tissue in tumor burden zones, distal normal zones and interface zones between tumor and normal tissue differed with respect to their ability to induce the epithelial-mesenchymal MCF7 cell transition, modulation of the membrane-type 1 matrix-metalloproteinase expression, activation of the ERK signa-

ling cascade and promotion of the cancer cell migration [30]. The fibroblasts isolated from the tumor interface zone exhibited more robust biological modulatory activity and promoted the breast cancer cell migration. This suggests that the interface zone of the tumor represents a dynamic region vital to the tumor progression [30].

The presence of eEF1A2 in the cancer samples of different tissues has been well documented [18–25], but the molecular events underlying this phenomenon are still not understood. We can speculate that the expression of *EEF1A2* gene in the normal tissues surrounding the tumor may be also somehow induced by cancer cells. The molecular mechanism of such induction remains to be investigated.

Conclusions. The anti-peptide antibodies that specifically recognize eEF1A2, but not eEF1A1, were obtained after goat immunization and affinity chromatography purification. These antibodies were used for the determination of eEF1A2 protein in both the crude cell extracts and the postoperative tumor samples. We confirmed the presence of this protein in tissue specimens of human lung, breast and stomach malignant tumors by immunoblotting and immunohistochemistry. The presence of eEF1A2 in the samples of normal tissue raises new questions concerning determination of the tumor boundaries and its influence on the adjacent tissues.

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Authors declare a conflict of interests as inventors (V. F. Shalak, O. V. Novosylna, E. F. Kolesanova, E. A. Egorova, A. A. Mishin, B. S. Negrutskii) of the patent application related to the synthetic peptide antigen for the preparation of monospecific antibodies against eEF1A2.

Характеристика нових пептидоспецифічних антитіл проти фактора елонгації трансляції eEF1A2 та їхнє використання для дослідження зразків пухлин

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Резюме

Мета. Охарактеризувати особливості нових пептидоспецифічних антитіл проти другої ізоформи фактора елонгації трансляції 1A (eEF1A2). Визначити присутність цієї ізоформи у післяопераційних зразках пухлин молочної залози, легенів, шлунку людини і порівняти із зразками умовної норми відповідних тканин. **Методи.** Специфічність антитіл аналізували методами імуноферментного аналізу, імуноблотингу та імуногістохімії. Імуноблотинг та імуногістохімію використано для визначення eEF1A2 в зразках пухлин людини, а також умовної норми. **Результати.** Встановлено, що отримані антитіла проти eEF1A2 специфічно розпізнають зазначений білок в екстрактах та на гістологічних зрізах і не виявляють перехресної взаємодії з першою ізоформою фактора елонгації. Визначено, що фактор eEF1A2 присутній в післяопераційних зразках пухлин молочної залози, легенів і шлунку, а також в умовно нормальних зразках цих тканин. **Висновки.** Нами одержано високоспецифічні антитіла проти eEF1A2, які можна використовувати в імунологічних дослідженнях зразків пухлин.

Ключові слова: eEF1A2, антипептидні антитіла, імуноблотинг, імуногістохімія.

Характеристика новых пептидоспецифических антител против фактора элонгации трансляции eEF1A2 и их использование для изучения образцов опухолей

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Резюме

Цель. Охарактеризовать особенности новых пептидоспецифических антител против второй изоформы фактора элонгации трансляции 1A (eEF1A2). Определить наличие этой изоформы в послеоперационных образцах опухолей молочной железы, легких, желудка человека и сравнить с условной нормой соответствующих тканей. **Методы.** Специфичность антител анализировали методами иммуноферментного анализа, иммуноблотинга и иммуногистохимии. Методы иммуноблоттинга и иммуногистохимии использовали для определения eEF1A2 в образцах опухолей человека, а также условной нормы. **Результаты.** Установлено, что полученные антитела против eEF1A2 специфически распознают этот белок в экстрактах и на гистологических срезах и не выявляют перекрестного взаимодействия с первой изоформой фактора элонгации. Мы определили, что фактор eEF1A2 присутствует в послеоперационных образцах молочной железы, легких и желудка, а также в условно нормальных образцах этих тканей. **Выводы.** Полученные нами антитела против eEF1A2 являются высокоспецифическими по отношению к антигену и могут быть использованы в иммунологических исследованиях опухолей.

Ключевые слова: eEF1A2, антипептидные антитела, иммуноблотинг, иммуногистохимия.

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