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GENERATION AND CHARACTERIZATION OF POLYCLONAL ANTIBODIES SPECIFIC TO HUMAN ESTROGEN RECEPTOR ERα

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Aim. The purpose of the study was to generate and characterize anti-hERα polyclonal antibodies for elucidation of functional relationships between isoforms of estrogen receptor ERα and isoforms of ribosomal protein S6 kinase — S6K1.

Methods. cDNA cloning. Expression of recombinant proteins in bacterial system. Affinity purification of His-tag fused recombinant proteins using Ni-NTA chromatography from bacterial lysates. Generation of polyclonal sera by mice immunization. Western blot analysis and immunoprecipitation.

Results. cDNA coding for full length hERα was cloned into expression vector pET28a in frame with His-tag sequence. Recombinant hERα-His protein was expressed in E.Coli and purified by Ni-NTA chromatography. Purified hERα-His was used as antigen for mice immunization and generation of polyclonal antibodies. Specificity of polyclonal antibodies was analyzed by Western blot and immunoprecipitation of hERα from MCF-7 cell lysates.

Conclusions. Generated anti-hERα polyclonal antibodies are of conformational type since specifically recognized hERα only in immunoprecipitation but not in Western blot. Created polyclonal antibodies a suitable for detection and analysis of hERα protein complexes.

Key words: human estrogen receptor α (ERα), cloning, expression of recombinant proteins, antibodies, immunoprecipitation.

Breast cancer (BC) is a common type of malignancy among females, worldwide [1]. The generally accepted classification of breast tumors is based on the expression of estrogen (ERα), progesterone (PR) and epidermal growth factor (HER2) receptors. Tumors of luminal subtypes that are ERα+ have a more favorable prognosis of the course of the disease and response to antitumor therapy compared to ERα negative and especially triple-negative subtypes, which are the most aggressive and characterized by a high level of metastasis and, accordingly, a low patient survival rate [2]. The most prevalent breast cancer subtype is hormone receptor positive, expressing the ERα and/or progesterone receptor, accounting for approximately 75% of breast cancers [3, 4]. ERα functions as a ligand-dependent transcription factor that regulates the expression of estrogen-responsive genes, stimulates cancer cell proliferation and contributes to tumor progression [5]. Binding with estrogens in the cytoplasm of the cell induces the formation of a ERα homodimeric complex and translocation to the nucleus that cause initiation of transcription of genes containing the estrogen response element (ERE) in the promoter regions. Another activity of ERα is linked to receptor location on the cell membrane, where it performs the function of a modulator of signaling cascades, including the mTOR/Akt/S6K-dependent cascade, which performs the function of the main integrator of extracellular stimuli in the cell, controlling the main cellular functions. Therefore it is one of the key factors in the regulation of tumor progression [6]. Endocrine therapies, which suppress the ERα signaling pathway, significantly improve the prognosis of patients with breast cancer. However, the development of *de novo* or acquired endocrine therapy resistance remains a barrier to breast cancer treatment [7]. Due to the prevalence of ER+ breast tumors the standard for use

in the clinic is anti-estrogen therapy, that is based on application of estrogen antagonists, negative regulators of ERα expression and estrogen synthesis blockers. However, the response to such therapy ranges from 35% to 70%, and in many cases, the development of resistance to the therapy is observed over the time. The mechanism of such resistance is not known for sure. One of the reasons may be the ligand-independent activation of the main p66 isoform of ERα and suppression of its expression, as well as activation of the oncogenic p36 isoform of ERα by an unknown mechanism [8].

The role of ribosomal protein S6 kinase — S6K1 in disease development and progression is supported by the observation that S6K1 is overexpressed in breast cancer and it is associated with poor prognosis of cancer patients [9-11]. However, the identity of mammary cell-specific S6K1 targets is not well understood. It should be noted that S6K1 regulates ERα by phosphorylating it on serine 167, leading to transcriptional activation of ERα. By contributing to the activation of ERα, S6K1 promotes ERα-mediated cell proliferation and may be a target of therapeutic intervention in breast cancer [12].

Recently, we identified a new p60 isoform of S6K1, which significantly differs in regulation from the known p70 and 85 kinase isoforms [13–15], namely that is not sensitive to mTOR inhibitors used in combination with ERα inhibitors for BC treatment. In addition, convincing data were obtained regarding the involvement of S6K1 isoforms in the regulation of ERα expression in BC cells. According to our data, selective inhibition of the expression of p70 and p85 S6 kinase isoforms is accompanied by dramatic changes in the expression of ERα, up to complete blocking, which accompanies the initiation of EMT inherent for triple negative subtypes of BC [16]. Therefore, elucidating the relationship between the regulation of activity and expression of ERα and S6K1 isoforms will contribute to a deeper understanding of their role in breast carcinoma cells, taking into account tumor molecular subtypes, and therefore to the development of new treatment strategies for both ER^+ and ER^- subtypes of BC.

Research in this direction requires elucidating the intermolecular interactions of S6K1 and ERα isoforms by immunoprecipitation of protein complexes from the lysates of model breast cancer cells using antibodies against ERα and/or S6K1. We believe that different parts of ERα would be involved in interaction with different protein partners including isoforms of S6K1 and so the application of polyclonal antibodies instead of monoclonal would be more preferential for isolation of protein complexes.

The aim of this studies was to obtain and characterize polyclonal antibodies specific for ERα that would be suitable for studies of functional relationships between isoforms of estrogen receptor ERα and its different protein partners.

Materials and Methods

Generation and purification of recombinant hERα-His. cDNA fragment encoding the full-length (1-595 aa) human ERα was amplified by PCR using specific oligonucleotide primers (Forward 5′-ATAGGATCCATGACC-ATGACCCTCCACACCAAAGC-3′, Reverse — 5′-ATACTCGAGTCAGACCGTGGC-AGGGAAACCCTCTG-3′) and cloned into the expressing bacterial vector pET28a at the BamH1 and Xho1 sites in the frame with the N-terminal polyhistidine (6His-tag) sequence. As a template we used cDNA synthesized from mRNA of MCF-7 cells (16). The primary structure of the cloned cDNA was verified by sequencing. pET28a/ERα-His was transformed into *Escherichia coli* strain "Rozetta" and the expression of recombinant ERα-His was induced with 1 mM IPTG for 3 h at 37 °C in presence of 10% sucrose. Affinity purification of recombinant hERα-His from bacteria cell lysates was carried out on Ni-NTA agarose ("Qiagen", USA) at denatured conditions according to the manufacturer's recommendations. The purity of the hERα-His was analyzed by SDS-PAGE.

Generation of polyclonal antiserum. Female BALB/c mice (8 weeks old) were immunized with 15 μg of recombinant ERα-His protein in PBS with 50% complete Freund's adjuvant using intraperitoneal injection (i.p.). Subsequent immunizations were carried with 15 μg of recombinant protein with 50% incomplete Freund's adjuvant by i.p. injection at 2-week intervals until the titer of anti-hERα-His antibodies in blood sera reach 10^{-5}. The titer of antibodies was monitored by ELISA. Two weeks after the last immunization mice were boosted with 15 μg of antigen by i.p. injection in PBS without adjuvant. Three days later, blood from immunized mouse was collected for anti-sera preparation that was further diluted by glycerol up to 50% for storage.

Western blot analysis. Lysate of MCF-7 cells was prepared using lysis buffer

containing 15 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 1 mM DTT, 1 mM EDTA, and a mixture of protease inhibitors (Roche Molecular Diagnostics, Meylan, France). Cell lysates (25 μg) were separated by SDS-PAGE and transferred onto Immobilon-P membrane (Millipore, Billerica, MA). The membrane was blocked with 5% nonfat milk in TBST (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.05% Tween-20) for 30 min. at rotation, and then incubated with anti-ERα-His polyclonal sera in 1:1000 dilution over night at +4 °C. Following with three times 10 min. washing with TBST, membrane was incubated 1h at rotation with HRP-conjugated goat anti-mouse IgG in 1:10000 dilution (Jackson Immuno Research Laboratories, Pennsylvania, USA). After second round of washing the membrane was developed using an enhanced chemoluminescence (ECL) reaction and then exposed to Agfa X-ray film.

Immunoprecipitation. Cell lysate (500 μg) was incubated with 5 μl of ERα anti-sera and 30 μl of 50% slurry of protein G-Sepharose (GE Healthcare) in a total volume of 500 μl lysis buffer at rotation at 4 °C overnight. Protein G Sepharose with bound to the beads immune complexes were recovered by centrifugation with following 10 min washing three times with 1 mL of lysis buffer. The washed beads were boiled for 5 min in 40μl Laemmli sample buffer and analyzed by western blot using corresponding antibodies.

Results and Discussion

For the analysis of ERα functional complexes with S6K,1 as our main interest, and the other protein partners as well using immunoprecipitation approach, we aimed to generate polyclonal antibodies specific to human ERα. In addition to already mentioned reason we were prompted to do so by the fact that commercially available anti-ERα antibodies differ significantly among themselves in terms of their specificity. The data of Western blot analysis cell lysates of MCF-7 and MCF-7 ($p70^-/p85^-/p60^+$/S6K1) subline with suppressed expression of p70 and p85 isoforms of S6K1 allowed us to draw this conclusion. According to the PCR analysis ERα gene expression was detected at a high level in MCF-7 cells, but it was completely blocked in MCF-7 subline $p70^-/p85^-/p60^+$/S6K1 (16). However, of the two types of commercial antibodies tested, only one confirmed the data of PCR analysis on the protein level (Fig. 1). Thus, antibodies positioned as anti-hERα that have no difference in ERα recognition in two MCF-7 cell lines may have a different specificity or may recognize an yet unknown isoform of ERα. Indeed, we have demonstrated that this type of antibodies recognizes a protein of very close, but lower molecular weight than previous antibodies (Fig.1, line 1, 2)

To generate anti-hERα polyclonal antibodies as an antigen for immunization we used full length hERα-His expressed in bacteria. For this on the initial stage we cloned DNA sequence corresponding to the full length ERα into pET28a vector in frame with the 6xHis sequence. Generated pET28a/ERα-His plasmid was used for transformation of *Escherichia coli* strain "Rozetta". Initially expression of the recombinant protein was at very low level (data not shown), however addition of 10% sucrose to the growing media dramatically increases the expression of recombinant protein that was insoluble (Fig. 2, *A*).

Purification of ERα-His was performed using Ni-NTA affinity chromatography at denatured conditions in presence of 8M urea (Fig. 2, *B*). According to the data of Fig. 2, *B* ERα-His expressed in *E. coli* is subject to proteolytic cleavage (Fig. 2, *A, B*). One can see that 1mM IPTG induce expression of two protein bands that corresponds to the full length of ERα-His (upper band) an its truncated form (Fig. 2, *A*). Important, that both forms are the subject of affinity purification on Ni-NTA beads (Fig. 2, *B*) and are recognized by the commercial anti-hERα

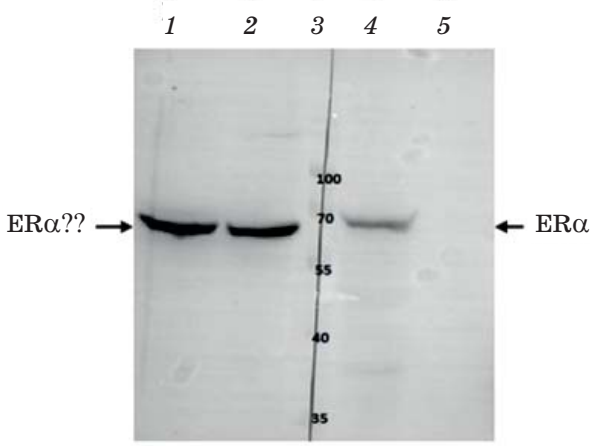

Fig. 1. **Western blot analysis of MCF-7 and MCF-7 subline $p70^-$, $p85^-$, $p60^+$/S6K1 cell lysates with different anti-ERα mAbs**

Lanes *1, 2* — mouse anti-ERα mAbs (Abcam, MA5-15689); lanes *4, 5* — rabbit anti-ERα mAbs (Abcam, MA5-14501). *1, 4* — MCF-7 lysates (20 μg); *2, 5* — MCF-7 ($p70^-$, $p85^-$, $p60^+$/S6K1) lysates (20 μg); *3* — protein markers with molecular weight indication in kDa

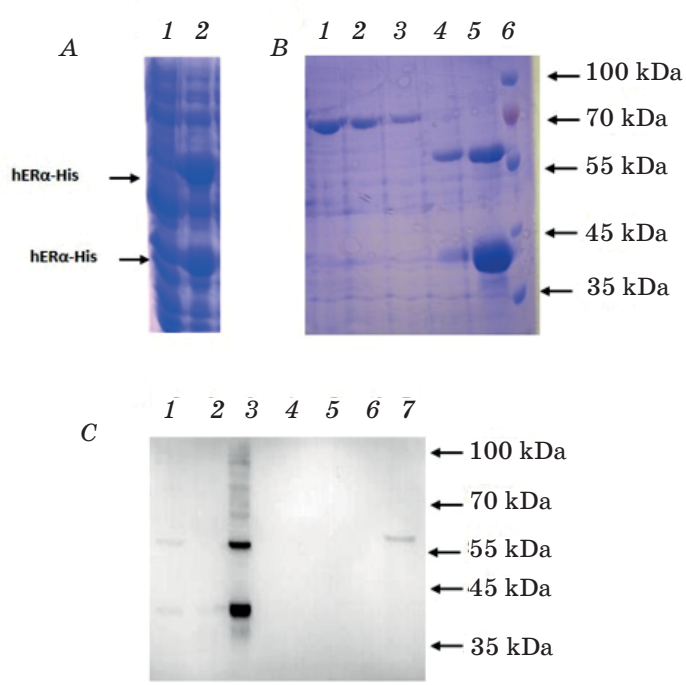

Fig. 2. **Expression of recombinant hERα-His in *Escherichia coli* transformed with pET28a/hERα**
A — SDS-PAAG electrophoresis of bacterial cell lysates (30 μg) before (*1*) and after IPTG induction (*2*); *B* — SDS-PAAG electrophoresis of purified by Ni-NTA chromatography hERα-His: *1–3* — BSA; *2* — 1–0,5 μg; *4* — the first elution (5 μl); *5* — the second elution (5 μl); *6* — protein markers; *C* — Western blot analysis of hERα-His with anti-hERα mAbs (Abcam, MA5-14501): *1* — bacterial cell lysate (10 μg) before IPTG induction; *3* — bacterial cell lysate (10 μg) after 1 mM IPTG induction; *4* — protein markers; *5–6* — BSA 1–0,5 μg; *7* — lysate of MCF-7 cells (15 μg)

antibodies (Fig. 2, *C*). In addition, we saw a change in the ratio between the full-length and truncated forms towards an increase in the content of the truncated form, which indicates protein degradation during the purification process as well (Fig. 2, B). Taking into account that both ERα forms are recognized by anti-hERα mAbs (Abcam, MA5-14501) generated to the C-terminal peptide of hERα we assume that the proteolytic cleavage occurs at the N-terminus of hERα.

The purified recombinant full size ERα was used for further mice immunization according to the presented protocol. Immunizations were continued until the blood serum titer of anti-ERα antibodies tested by ELISA reached 10^{-5}.

Initially obtained anti-ERα serum of immunized mice was analyzed by western blot using MCF-7 cell lysates. Unfortunately generated anti-ERα polyclonal antibodies did not recognize an antigen (data not shown) in denatured state.

Next, we investigated whether the obtained antibodies are able to recognize ERα in the immunoprecipitation assay by recognizing conformation dependent antigenic epitopes. As a control, immunoprecipitation of ERα was performed from cell lysate using the commercial antibodies (Abcam, MA5-14501). According to the data received anti-ERα polyclonal antibodies efficiently precipitated ERα that was further detected by western blot using commercial mAbs with confirmed specificity (Fig. 3).

Noteworthily, but both types of antibodies (commercial and just generated) precipitated two protein bands of ERα with only one matched to the band recognized in total cell lysate. Additional protein band with higher molecular weight may represent modified and minor form of that is recognized by anti-ERα/full polyclonal antibodies in immunoprecipitation even more efficiently then the mainly recognized in total cell lysate by Western blot ERα form.

For further verification of anti-ERα antibodies specificity we analyzed if their immunoprecipitates is recognized by anti-ERα antibodies that recognized so far unknown protein band with molecular mass close to ERα in Western blot (Fig. 1). According to the data of fig. 4 the mentioned commercial anti-ERα mAbs (Abcam, MA5-15689) did not recognized immunoprecipitated ERα

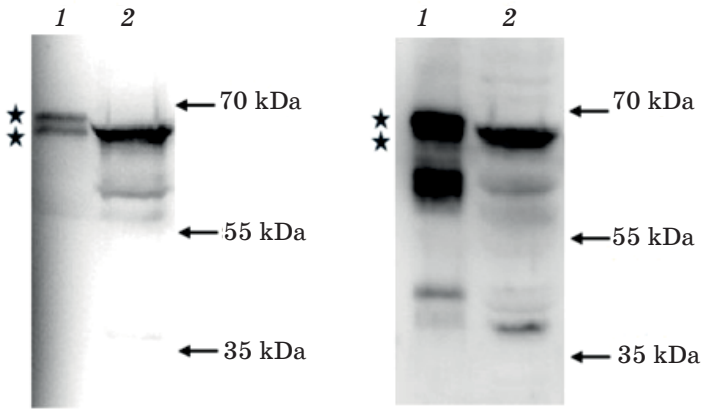

Fig. 3. **Western blot analysis of ERα immunoprecipitated from MCF-7 cell lysates by different types of anti-ERα antibodies and developed by the rabbit anti-ERα mAbs (Abcam, MA5-14501)**
A — ERα immunoprecipitated by the polyclonal mouse anti-ERα/full antibodies; *B* — ERα immunoprecipitated by rabbit anti-ERα mAbs (Abcam, MA5-14501); *1* — immunoprecipitate of ERα from MCF-7 lysates; *2* — MCF-7 total cell lysate (25 μg)

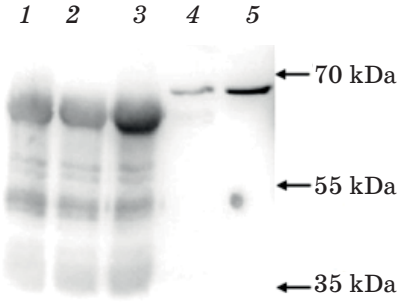

Fig. 4. **Western blot analysis of S6K1 immunoprecipitated by the polyclonal mouse anti-ERa/full:**
MCF-7 (*1*) and MCF-7 ($p70^-$, $p85^-$, $p60^+$/S6K1) (*2*) cell lysates and developed by mouse mAbs (Abcam, MA5-15689); *3* — control precipitation with no lysate; *4* — lysate of MCF-7 (25 μg); *5* — lysate of MCF-7 ($p70^-$, $p85^-$, $p60^+$/S6K1) subline (25 μg)

confirming specificity of generated polyclonal anti-ERα antibodies.

Conclusion

As a conclusion, using full size hERα-His, expressed in bacteria and purified by affinity chromagraphy on Ni-NTA Sepharose, as an antigen for mice immunization we generated polyclonal anti-hERα antibodies that are suitable for efficient immunoprecipitation of ERα and so suitable for studies of hERα involved in protein complexes formation.

Author Contributions

A.M. — Western blot analysis, immunoprecipitation, I.K.- DNA cloning, protein expression and purification, animal immunization.

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СТВОРЕННЯ ТА ХАРАКТЕРИСТИКА ПОЛІКЛОНАЛЬНИХ АНТИТІЛ СПЕЦИФІЧНИХ ДО ЕСТРОГЕНОВОГО РЕЦЕПТОРА ЛЮДИНИ ERα

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Мета. Отримати та охарактеризувати поліклональні антитіла проти ERα для подальшого з'ясування функціональних зв'язків між ізоформами рецептору естрогену ERα та ізоформами кінази рибосомного протеїну S6 — S6K1.

Матеріали та методи. Клонування кДНК. Експресія рекомбінантних протеїнів у бактеріальній системі. Афінне очищення з бактеріальних лізатів рекомбінантних протеїнів злитих з His-tag за допомогою Ni-NTA хроматографії. Імунізація мишей. Вестерн-блот аналіз та імунопреципітація.

Результати. кДНК, що кодує повнорозмірнуформу hERα клонували у вектор для експресії pET28a в рамці з His-міткою. Рекомбінантний протеїн hERα-His експресували в *E. coli* та очищали Ni-NTA хроматографією з клітинного лізату. Очищений hERα-His використовували для імунізації мишей і генерації поліклональних антитіл, специфічність яких аналізували вестерн блот аналізом та імунопреципітацією hERα з лізатів клітин лінії MCF-7.

Висновки. Створені анти-hERα поліклональні антитіла мають конформаційний тип, оскільки специфічно розпізнаються hERα в імунопреципітації, але не в вестерн-блоті. Створено поліклональні антитіла, придатні для виявлення та аналізу протеїнових комплексів hERα.

Ключові слова: естрогеновий рецептор α людини (ERα), клонування, експресія рекомбінантних протеїнів, антитіла, імунопреципітація.