Obtaining Specific Hybridomas for Ki-67 Protein Immunodetection

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Abstract

BACKGROUND: Active proliferation is specific property of a tumor cells. However, the cost of the analysis is high due to commercial anti-Ki-67 mAbs used as the main immunoreagent for reliable identification of proliferating cells. In this study, recombinant protein was used to obtain specific mAbs for Ki-67 biomarker immunodetection.

METHODS: Codon optimized fragment of *ki-67* gene was cloned into the pET28c(+)vector. The recombinant protein was purified by immobilized metal affinity chromatography (IMAC) and confirmed by liquid chromatography–mass spectrometry (LC-MS)/MS. Hybridoma cells were obtained by fusing myeloma cells with mouse spleen cells immunized with recombinant antigen. The specificity and activity of mAbs was determined by enzyme-linked immunosorbent assay (ELISA), Western blot and immunocytochemistry.

RESULTS: The pET-28c(+)/*ki-67* plasmid, which encodes 355 amino acid protein, was obtained. Analysis by LC-MS/MS of the recombinant antigen showed that 77.5% of the amino-acid sequence belonged to Ki-67 protein. Recombinant fragment of Ki-67 protein was used to obtain specific hybridoma strains. ELISA and Western blot demonstrated high affinity and the specificity of obtained mAbs against Ki-67 protein. Newly generated anti-Ki67 mAbs detected target protein in proliferating cells of MCF-7 cell line by immunocytochemistry.

CONCLUSION: Newly developed mAbs are potentially useful as an immunodiagnostic tool for assessing the proliferative activity of breast tumor cells using immunocytochemistry.

KEYWORDS: breast cancer, Ki-67, monoclonal antibodies, nuclear antigen, recombinant antigen, tumor cells

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Introduction

Breast cancer is a leading malignant disease among women in many countries, despite its pathology is visually localised. High heterogeneity of breast cancer determines the study of pathological changes at the molecular level.(1-3) Every year in the world over two million women are diagnosed with breast cancer. In 2020 in Kazakhstan breast cancer ranks the first place in the total number of detected cases of cancer patients to 37.1 % of all 35366 registered cases.(4)

Early diagnosis of breast cancer determines the clinical course and prognosis of the disease.To confirm the pathology of the mammary glands using cytomorphological diagnostics by fine needle aspiration cytology (FNAC). However, the reliability and accuracy of the cytological study varying from 34 to 65%.(5) In the recent years, FNAC technique is improved by the development immunocytochemistry (ICC) based to determine the molecular biomarkers of tumors. Immunocytochemical characterization of breast carcinoma on fine-needle aspirates (FNA) is recognized as an effective method study the prognostic and predictive molecular biomarker of tumor cells for early diagnosing of malignancy. Assessment of status in lymph nodes from patients receiving neoadjuvant therapy or patients with inoperable metastases in FNA

samples using ICC has been successful to predict the course of the treatment.(6-9)

As known active proliferation is specific property of a tumor cells population. At present, detection of cell proliferation index using the Ki-67 biomarker is widely used as potential prognostic biomarker in breast carcinoma in diagnostic routine clinical practice.The Ki-67 biomarker is mainly associated with chromosomes and belongs to the family of perichromonucleic proteins that surround chromosomes during mitosis.(10) The Ki-67 nuclear antigen is present in the G1, S, G2, and mitotic phases of the cell cycle, but is not present in G0. Starting from the middle of the G1 phase, Ki-67 expression gradually increases, reaches a peak in metaphase during mitosis, and decreases during anaphase. The Ki-67 antigen is a regulatory cell-cycle protein that is vital in mitosis.(11-13) Thus, the presence of Ki-67 in nearly all phases of the mitotic cycle, and its disappearance upon transition into the resting period, renders it an optimal marker for assessing cellular proliferation.

The immunocytochemistry in FNA samples with biomarkers Ki67 showed it is possible to differentiate in the identification of benign and malignant lesions. Determination of Ki67 biomarker on FNA samples by immunocytochemistry is recommended for the screening and early diagnosis of breast cancer.(5,14) After the discovery of the Ki-67 full structure and the construction of its domains and subdomains using methods of genetic engineering, it became possible to obtain monoclonal antibodies (mAbs) with an epitope-specific orientation. (15) However, the cost of the analysis of is very high due to used commercial anti-Ki-67 mAbs as the main immunoreagent for reliable identification of proliferating cells. In this study, recombinant fragment of Ki-67 protein was used to obtain specific mAbs that allow to reliably identify the Ki-67 biomarker. Obtained specific mAbs can be used to evaluate the proliferative activity of tumor cells using immunocytochemistry (ICC) in FNA samples for the screening and early breast cancer diagnostics.

Methods

Cloning and Expression of the *ki-67* Gene Fragment in *Escherichia coli* and Purification of Recombinant Ki-67 Protein
Gene sequence of human *ki-67* was derived from the National Center for Biotechnology Information (NCBI) Genbank (NM_001145966.2) and used to engineer a synthetic gene encoding protein fragment (amino acid region at 1160-1493). To optimize the expression of recombinant eukaryotic Ki-67 protein in prokaryotic *E. coli* cells, the codon optimization of target gene fragment was performed through a table of *E. coli* frequently used codons. (16) The optimized sequence was verified by GenScript online service (*https://www.genscript.com/tools/rare-codon-analysis)*. A resulting 1002 bp gene sequence was synthesized and provided as pTopBluntV2/*ki-67* plasmid by Macrogen (Seoul, South Korea).

The fragment of *ki-67* gene was cloned into the pET28c(+) expression vector with primers NdeI-fw-Ki67 (5'-GGGAATTCCATATGTTCCTGGCGCTGAGGAA AC-3') and BamHI-rv-Ki67 (5'-CGCGGATCCTTACG CGATTTTGGTTGTTTTTTC-3'). The plasmid insert was sequenced using BigDye Terminator v. 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730xl sequencer (Applied Biosystems, Foster City, CA, USA).

A single colony of recombinant *E. coli* strain BL-21 (DE3)/pET28c(+)/*ki-67* was incubated in 1L LB broth with kanamycin (50 μg/mL) (Sigma-Aldrich, Taufkirchen, Germany) at 37°C, 200 rpm upon reaching OD_{600}=0.6. The culture was induced by 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG; AppliChem Lifescience, Darmstadt, Germany) at 37°C, 200 rpm for 2 hours. The precipitate was resuspended in 10 mL 1% Triton X-100, 20 mM NaCl, and 20 mM Hepes-KOH pH 7.5, shaken (100 rpm, 22°C, 30 minutes) and centrifuged (18000×g, 4°C, 30 minutes).The pellet was resuspended in 10 mL 1M urea, 20 mM HEPES-KOH pH 7.5, and 10 mM beta-mercaptoethanol. This procedure was repeated using 3 M, 5 M, and 8 M urea. Supernatants were collected and pooled. The recombinant protein was purified by immobilized metal affinity chromatography (IMAC) using 2 mL Ni-NTA agarose (Qiagen, Germantown, MD, USA). The desired fractions were combined and dialyzed against phosphate buffered saline (PBS) for 24 hours. Samples were stored in 50% glycerol at -20ºC.

Liquid Chromatography Combined with Mass Spectrometry (LC-MS/MS)
Proteins were separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) using 4-12% gradient polyacrylamide gels. Candidate bands were excised from the gels, alternately treated with 50 mM ammonium bicarbonate and 100% acetonitrile. Activated by 190 mM ammonium bicarbonate trypsin (Promega, Madison, WI, USA) was added to the samples. The peptide mixture was purified by ZipTipC18 (Millipore, Darmstadt,

Germany) and dried in a vacuum concentrator (35°C, 30 minutes). The precipitate was suspended in 10 µL 0.1% trifluoroacetic acid for mass spectrometry analysis.

The peptides were separated by high performance liquid chromatographic using an Acclaim PepMap100 C18 capture column (5 mm x 300 µm; particles 5-µm in diameter; Thermo Scientific, Austin, TX, USA) and Dionexpump (Thermo Scientific, Austin, TX, USA). Peptides were further separated on an Acclaim PepMap RSLC column (15 cm x 75 µm, particles 2-µm in diameter; Thermo Scientific, Austin, TX, USA). An unmodified Captive Spray ion source (capillary voltage of 1300 V, dry gas flow of 3 L/min, and dry temperature of 150°C) was used to interface the liquid chromatography (LC) system with Impact II. Mascot software was used to search the Swiss Prot 2014_08 database (546238 amino acid sequences; 194363168 amino acid residues). Search parameters were set as follows: modification variables, oxidation (M); fragment ion mass tolerance, 20 ppm; parent ion tolerance, 20 ppm.

Production of mAbs Against Ki-67 Protein

Studies were conducted on 6 BALB/c mice (The Jackson Laboratory, Bar Harbor, ME, USA), 6-8 weeks old females, weighing 20-25 g. Animals were obtained from the Animal facility of the National Center for Biotechnology (NCB), Nur-Sultan city, Kazakhstan. All procedures involving animals were conducted in accordance with the guidelines of NCB and approved by Institutional Animal Care and Use Committee (protocol #2 June 2, 2020). Animals were housed in special facility with 12-hour light dark cycle. They were fed by standard mouse chow, water and food provided *ad libitum*.

On the first day of immunization, BALB/c mice (6-8 weeks old) were intraperitoneally injected with 0.05 mg recombinant Ki-67 protein emulsified in 0.1 mL of Freund's complete adjuvant. On days 7, 11, 12, 13 of immunization, were injected with 0.025 mg of antigen in. of PBS pH 7.2. Hybridization of myeloma cells X63Ag8.653 (ATCC, Manassas, VA, USA) with spleen cells from immunized mice was performed according to the method of Oi and Herzenberg.(17) The fusion agent was used 45% solution of polyethylene glycol-4000 (PEG; Merck, Darmstadt, Germany) and as selective HAT (hypoxanthine, aminopterin, and thymidine) medium (Sigma-Aldrich, Saint Louis, MO, USA). The productivity of the hybrid cells determine by indirect enzyme-linked immunosorbent assay (ELISA) using recombinant protein Ki-67.

Single-cell cloning of hybrid cells was carried out using the limiting dilution method.(18) The concentration of mAbs in ascitic fluid was determined using Bradford assay. (19) Isotypes of mAbs were determined using a IsoStrip™ Mouse Monoclonal Antibody Isotyping Kit (Sigma-Aldrich,Taufkirchen, Germany).

Indirect ELISA

Indirect ELISA was performed by coating a 96-well plate with recombinant antigen Ki-67 at a concentration of 0.001 mg/mL in 0.1 mL of PBS (pH 7.2) and incubated overnight at 4°C. Primary antibody (blood sera of immunized mice, supernatant or ascitic fluid mAbs) was added to the wells and incubated for 1 h at 37°C. Further, rabbit anti-mouse secondary antibody (labeled with horseradish peroxidase) diluted 1:5000 in PBST. After of incubation a substrate 3,3',5,5'-tetramethylbenzidine (Sigma-Aldrich, Taufkirchen, Germany) was added. The absorbance of the final product was measured at 492 nm, using a spectrophotometer.

Western Blot

Western blot of recombinant Ki-67 protein was performed by standard method.(20) Obtained 4B8 mAbs were used as the primary antibodies at 1:500 dilution, 4°C overnight. Commercial anti-Ki67 mAbs B56 (550609, BD Pharmingen, San Jose, CA, USA) were used as positive control at 1:250 dilution. Goat anti-mouse antibodies conjugated with horseradish peroxidase (Sigma-Aldrich) were used as the secondary antibodies at 1:10000 dilution. The developing buffer includes the substrate solution, which contains 0.48 mM 4-Chloro-1-Naphthol (Sigma-Aldrich), 50 mM Tris-HCl and 0.2 M NaCl in 17% methanol, and 0.01 mL of 3% (v/v) hydrogen peroxide.

ICC

Breast adenocarcinoma cells MCF-7 (ATCC, Manassas, VA, USA) were passaged on culture slides at a density of 50000 cells/well until a monolayer was formed with a confluence index of 80%. After reaching the required level of confluence, cells fixed with 4% paraformaldehyde for 20 min at 37°C. After removing 4% paraformaldehyde, to permeabilize the cell membranes, cells were treated with 0.3% Triton X-100 for 20 min. After block nonspecific binding 1% BSA, the cells were incubated with primary antibodies, 4B8 mAbs, at dilutions of 1:50 - 1:100. Commercial B56 mAbs at 1:50 dilution was used as positive control. Subsequently, a secondary conjugated antibody, anti-mouse Alexa Fluor 488 (Abcam, Burlingame, CA, USA), was added at 1:500 dilution. Images were captured at 40X magnification in a fluorescent microscope (Axio Observer, Carl Zeiss, Heidelberg, Germany) using a green filter.

Results

Expression and Purification of Recombinant KI-67 Protein

Part of the central domain Ki-67 protein with 16 repeating elements (*i.e.*, "Ki-67 repeats" (amino acid region at 1160-1493) was selected as antigen to obtain mAbs. This protein fragment is encoded by 1002 bp (NM_001145966.2, region: 2812-5640). To optimize the expression of recombinant eukaryotic Ki-67 protein in prokaryotic *E. coli* cells, the codon optimization of *ki-67* gene was performed through a table of frequently used *E. coli* codons. The Codon Adaptation Index (CAI) of the obtained gene sequence was 0.82 relative to the optimal range (0.8 - 1.0). GC content of the gene was 53.71%, which fell within the required 30 - 70% range. The percentage of low-frequency codons, which should not exceed 30%, was 3%. The initial sequence of *ki-67* gene fragment, selected for the codon optimization, is shown in Supplementary 1.

The synthesized gene encoding the recombinant protein, which is identical to the reference protein, was cloned into a pET-28c(+) vector at the NdeI and BamHI sites. Amplified gene is presented in Supplementary 2. Genetic map of the resulting plasmid pET28c(+)/ki-67 is shown in Figure 1. The *ki-67* gene is controlled by the T7 promoter in pET28c(+)/*ki-67* plasmid, the resulting recombinant fragment of Ki-67 protein carries an N-terminal 6xHis-tag. The open reading frame of the vector encodes a recombinant protein that contains 355 amino acid residues and has an estimated molecular weight of 38.5 kDa.
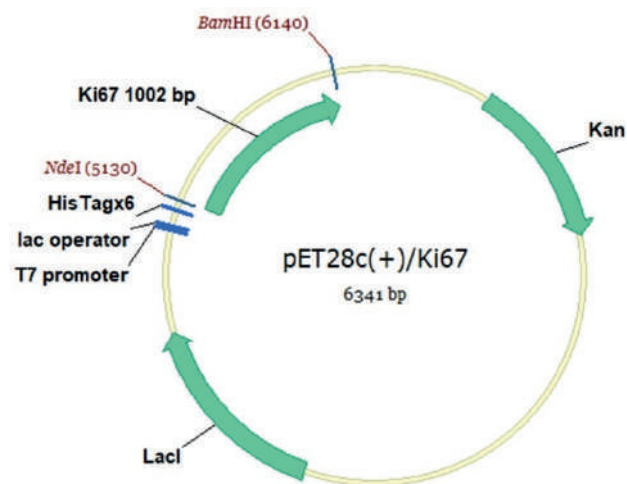

Figure 1. Genetic map of pET28c(+)/*ki-67* plasmid. The *ki-67* gene is controlled by the T7 promoter, the recombinant fragment of Ki-67 protein carries an N-terminal 6xHis-tag, the length of the open reading frame is 1068 bp.

Induction of protein expression for 16 hours did not lead to a significant increase in the amount of recombinant protein. SDS-PAGE showed protein accumulation at 45 kDa, which was ~6.5 kDa greater than the calculated mass of Ki-67 (Figure 2). Proteins with high proline content show reduced electrophoretic mobility because of bending and structural rigidity of the primary sequence. This can lead to an error of ± 10% in SDS-PAGE to determine the molecular weight of the protein. There are 32 proline residues in the structure of obtained recombinant Ki-67 protein, which may explain the observed mismatch in molecular weight obtained by electrophoresis. To verify our assumption, we analyzed the 45-kDa candidate protein by LC-MS/MS. We obtained a score of 10304 for peptides identified in the isolated protein, indicating that they belonged to Ki-67. Results of LC-MS/MS for identification of Ki-67 are presented in Supplementary 3. Analysis using Mascot program found peptides related to trypsin, keratin, and numerous other proteins. The presence of trypsin in the sample was likely due to porcine trypsin used in sample preparation. The presence of keratin peptides likely resulted from slight contamination during the experiment. The presence of other proteins may have been a coincidence in the m/z of primary and secondary ions, as confirmed by the low score. Analysis conducted by overlapping the identified peptides of the recombinant Ki-67 fragment showed that 77.5% of the amino-acid sequence belonged to Ki-67 protein (Figure 3). Cloned protein fragment is framed; identified peptides are bold. Protein sequence coverage in the recombinant Ki-67 protein fragment: 77.5%.

After verification of Ki-67 expression, a culture of the recombinant *E. coli* BL-21 (DE3)/pET28c(+)/*ki67* strain was developed for purification by IMAC (Figure 4A) The obtained fractions 1 and 2 were combined and used for the second step of purification (Figure 4B). Fractions 4-6 were combined and dialyzed against PBS for 24 hours. After dialysis the total protein yield was 47.22 mg from 1 L bacterial culture.

Production of the Hybridoma Strains

To immunize BALB/c mice with the recombinant protein Ki-67, the 2-week scheme was used. The optimal combination of five injections of the recombinant Ki-67 protein fragment produced sufficient amount of serum antibodies with a high titer for the antigen -1:102400–1:204800 (Figure 5). Specificity of interaction the recombinant Ki-67 antigen was confirmed using anti-Ki-67 mAbs named B56 as positive control and anti-human c-ErbB-2 mAbs as negative control (554299, BD Pharmingen). Our results indicate that
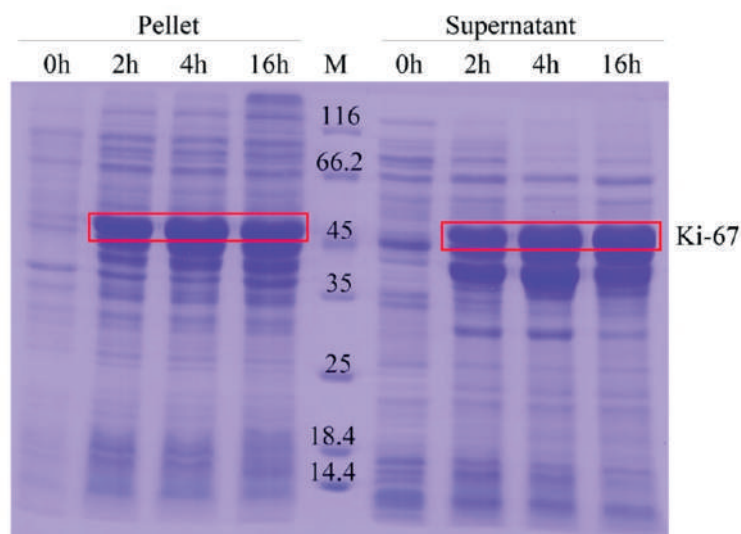
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Figure 2. Analysis of Ki-67 expression level in *E. coli* BL-21 (DE3)/pET28c(+)/*ki-67* cells. M: protein ladder (26610, Thermo scientific); 0h: no induction by IPTG; 2h: 2 hours of incubation after induction; 4h: 4 hours of incubation after induction; 16h: 16 hours of incubation after induction.

recombinant Ki-67 protein did not react with anti-human c-ErbB-2 mAbs, but it actively reacted with anti-Ki-67 B56 mAbs. Further, immunized mouse spleen cells were selected for hybridization with X63 myeloma cells.

Out of the total hybridomas seeded in 384 wells, growth of colonies was observed in 187-224 wells, *i.e.*, the percentage of cell fusion was high (48.6-58.3%).

Furthermore, the yield of positive clones obtained from total number of hybridomas was within 9.8% to 12.5%, indicating the possibility of isolating active clones with desired properties. Activity of hybridoma clones was examined five times by ELISA in an interval of 3-4 days.

Clones of 4B8, 7D2 and 10F5 cells, as the most active and stable lines of hybridomas, were cloned by the method

Figure 3. Overlap of the recombinant Ki-67 protein fragment with identified peptides by LC-MS/MS. Green frame: fragment of expressed protein; Red bold font: identified peptides.

A - Solubilization of the recombinant protein Ki-67 by Triton X-100 and urea

M - marker;
1 - 1% Triton X-100;
2 - 1 M urea;
3 - 3 M urea;
4 - 5 M urea;
5 - 8 M urea

B - Second step of the recombinant Ki-67 protein purification

M - marker;
1 - load;
2 - flow through;
3 - wash;
4 - 50 mM imidazole fraction;
5 - 100 mM imidazole fraction;
6 - 200 mM imidazole fraction;
7 - 300 mM imidazole fraction;
8 - 400 mM imidazole fraction;
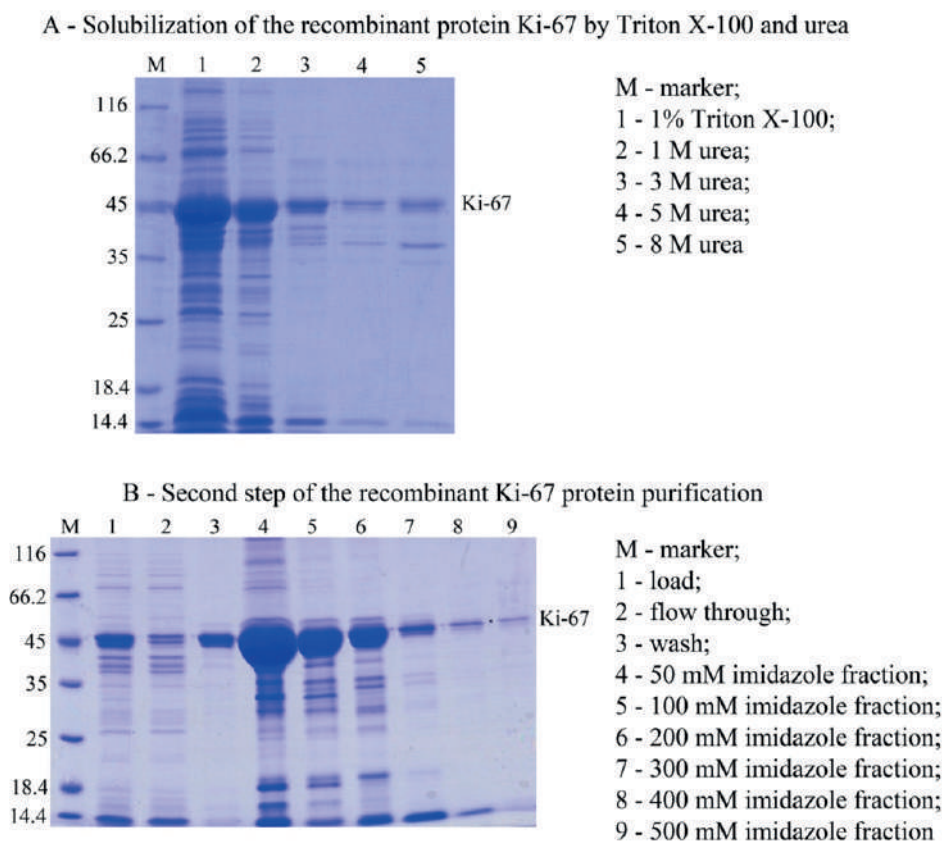9 - 500 mM imidazole fraction

Figure 4. Purification of the recombinant protein Ki-67 by IMAC.

of limiting dilutions. We found in genetically homogeneous or monoclonal cell population, as 85.9% to 94.4% of the subclones synthesized immunoglobulins specific to the recombinant Ki-67 protein fragment.

mAbs from the ascitic fluid were salted out using a saturated solution of ammonium sulfate. Purification of mAbs was performed by affinity chromatography, using

Figure 5. Antibody titer of sera from immunized mice. A,B,C: sera from immunized mice (n=3); D: commercial anti-Ki-67 mAbs; E: commercial anti-c-erB-2 mAbs; F,G,H: sera from non-immunized mice (n=3).

MabTrap Kit columns (GE Healthcare Life Sciences, Uppsala, Sweden). The *in vivo* cultivation of hybridoma cells led to significant increase in the concentration of mAbs to 2-4 mg/mL in the ascitic fluid, whereas the cultivation under in vitro conditions was 60-125 μg/mL. It was found that the hybridoma-synthesized antibodies were related to IgG class, the most active class of immunoglobulins.

The Specificity and Activity anti-Ki-67 mAbs

The specificity and activity of the hybridoma strains (4B8, 10F5, and 7D2) that produced mAbs were determined by indirect ELISA (Table 1). According to the results obtained from indirect ELISA, highest specificity to recombinant Ki-67 antigen showed 4B8 mAbs both in the culture fluid (1:512) and in the ascitic fluid (1:204800). However, the activity another of 7D2 mAbs to recombinant Ki-67 antigen was lower, which amounted to 1:64 in the culture fluid and 1:51200 in the ascitic fluid. The tested mAbs did not interact with extracellular domain of the recombinantc-ErbB-2 (the epidermal growth factor receptor of tumor cells).

The specificity of the isolated mAbs of the hybridoma strains was confirmed using a commercial mAb, anti-Ki-67 B56, as a positive control. Same levels of titers the experimental mAbs and commercial mAbs indicated that

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Table 1. Indirect ELISA to study the specificity and activity of mAbs of 4B8, 10F5, and 7D2 hybridoma strains.

Antigen Name	Monoclonal Antibody Titers			
	4B8	7D2	10F5	mAbB56
Recombinant fragment of Ki-67 protein	(1:512)/(1:204800)	(1:64)/(1:51200)	(1:256)/(1:102400)	1:51200
Recombinant extracellular domain of c-ErbB-2	No reaction	No reaction	No reaction	No reaction

numerator: titers in the culture fluid; denominator: titers in the ascite fluid.

both the antibodies recognized identical epitopes of the recombinant Ki67 antigen.

The specificity of the hybridoma strain 4B8 mAbs and commercial anti-Ki-67 B56 mAbs to the analogous epitope of the recombinant protein Ki-67 was confirmed by western blot (Figure 6). In both cases western blot analysis revealed the presence of a protein with a molecular weight of 45 kDa, a recombinant fragment of Ki-67 protein. Among them, 4B8 clones with a titer of 1:204800 in ascitic fluid were selected as suitable for research.

Intense staining of the nuclei of breast adenocarcinoma cells MCF-7 was revealed by ICC analysis using mAbs of 4B8 hybridoma strain (Figure 7). Specificity of detection to Ki-67 protein confirmed identical staining of nuclei of MCF-7 cells of commercial B56 mAbs. Intranuclear localization of the detected antigen suggests that 4B8 mAbs specifically interact with the nuclear protein Ki-67- biomarker of cell proliferation.

Discussion

Detection of cell proliferation is widely used in biomedical routine clinical practice. The main area of Ki-67 antibody application is ICC and immunohistochemical (IHC) methods. In this study, we have successfully produced novel anti-Ki-67 mAbs to detect biomarker of cell proliferation, the Ki-67.

Cell proliferation as a fundamental biological process, as well as a specific property of a transformed cells population that determines of malignancy of disease.(21) Currently, the most informative, accessible and common method of evaluation of the cell proliferative activity level is the identification of the molecilar biomarker, nuclear protein Ki-67. Immunochemically positive reaction to Ki-67 shows that the cell is in the interval from the late G1 phase to the M phase inclusive.(11-13) The Ki-67 protein is mainly associated with chromosomes, is detected in the telomere and centromere regions and belongs to the family of perichromonucleic proteins surrounding chromosomes during mitosis.(10,12)

The primary transcript of human *ki-67* gene is alternatively spliced. Two transcripts with different inclusion of exon 7 encode two 320-kDa and 350-kDa protein isoforms. The nuclear Ki-67 protein has an amphiphilic structure: its C-terminus can bind DNA and its N-terminus has an affinity for the cytoplasm. Although its splice variants differ in their N-terminus, they contain identical C-terminal and central (*i.e.*, Ki-67 domain) regions. Largest Ki-67 domain composed of multiple repetitive elements consisting of 122 amino acids in length with 82-100% homology, "Ki-67 repeats".(10-12,22,23) The targeting to largest "Ki-67 repeats" domain of multiple repetitive elements, which is unique to the Ki-67 protein, determines the epitope specificity of the most used commercial mAb for Ki-67 detection in clinical
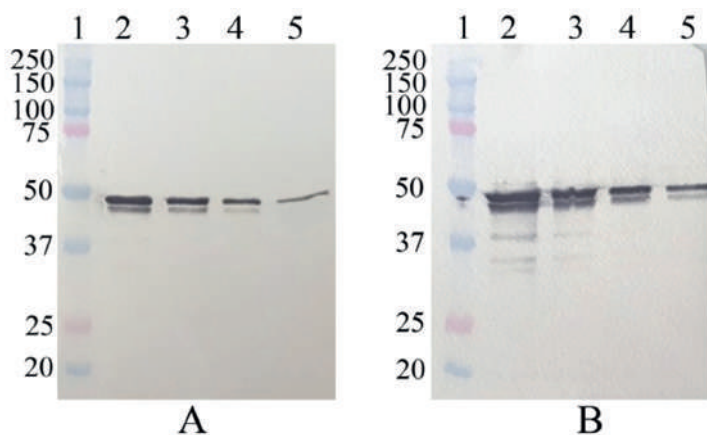

Figure 6. Western blot analysis of the specificity of commercial mAbs (A) and the hybridoma strain 4B8 mAbs (B) to recombinant protein Ki-67. 1: protein ladder; 2: 1 µg recombinant antigen (Ki-67); 3: 600 ng recombinant antigen (Ki-67); 4: 300 ng recombinant antigen (Ki-67); 5: 100 ng recombinant antigen (Ki-67).
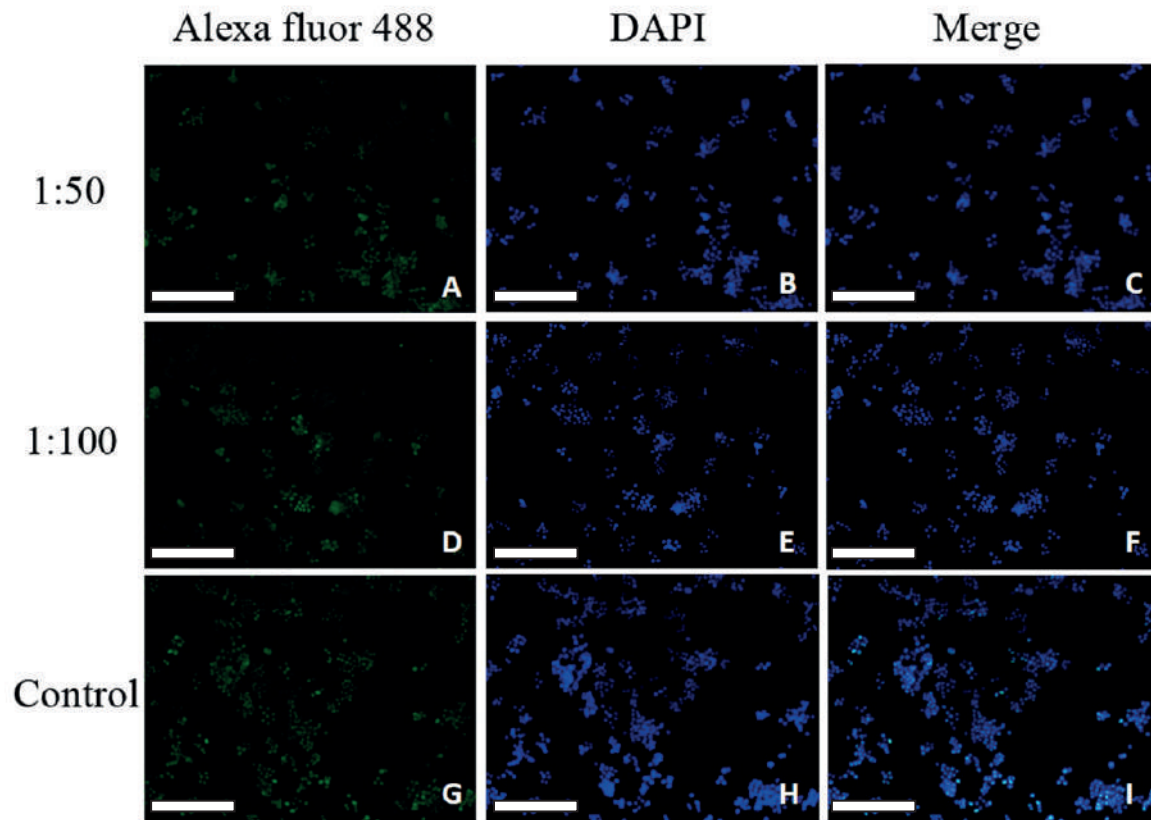
Figure 7. Immunocytochemical analysis of Ki-67 protein expression in breast adenocarcinoma cells MCF-7. A,B,C: 4B8 mAbs at 1:50 dilution; D,E,F: 4B8 mAbs at 1:100 dilution; G,H,I: commercial B56 mAbs at 1:50 dilution. Magnification 40X.

practice, MIB-1, 30-9 and SP6.(15,24-28) The synthesized *ki-67* gene fragment of recombinant Ki-67 protein with a length of 1002 bp (334 amino acids, region 1160-1493 aa) was used for production of specific anti-Ki-67 mAbs.The absence or low level of expression of heterologous protein is often observed in *E. coli* cells. In order to increase the expression level in *E. coli*, the nucleotide sequence of the human ki-67 gene was changed so that E. coli could easily recognize this sequence while maintaining the same amino acid sequence of the protein. The nucleotide sequence of the ki-67 gene has been optimized taking into account the relative codon frequency, GC content, the presence of hairpins and negative cis-regulatory elements that affect the expression level of a heterologous protein.(29) The codon optimized gene was synthesized and cloned into the vector pET-28c(+) for expression in *E. coli* BL-21 (DE3) cells. The yield of the recombinant protein was 47.22 mg/L due to the optimized induction parameters. It should be noted that the structural rigidity of the primary sequence reduced the electrophoretic mobility of the Ki-67 protein because of the high proline content.(30) As a result, SDS-PAGE showed a recombinant protein with a higher molecular weight than expected. The resulting protein was confirmed by LC-MS/

MS, 77.5% of the amino-acid sequence in the recombinant protein belonged to Ki-67.

Successful generation of hybridomas to produce mAbs is determined by the nature of immunogen and the efficiency of immunization in mice, which are the donors of immune B-lymphocytes that are hybridized with myeloma cells. The two-week scheme of immunization of BALB/c mice with the recombinant fragment of Ki-67 protein was suitable for stimulating the immune system of the organism. All immunized mice produced strong immune response with antisera titers above 1:102400, that considered as active B-lymphocyte clones producing specific antibodies in the spleens of immunized mice. These results suggested that recombinant Ki-67 protein expressed in prokaryotes elicited high immunogenicity in mice.

The first MIB-1 mAbs against the recombinant fragment of Ki-67 protein has been considered as the "gold standard" for immunochemical detection of this biomarker. Recent Ki-67 recognizing mAbs, SP6 and 30-9,were introduced as potential immunoreagents for routine clinical diagnostics.(15,31-33) According to the assessment international quality control shows that only two test systems based on the use of mAb MIB-1 (Dako) and 30-9

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(Ventana), has leading position in terms of laboratories use for assessing Ki-67 status and demonstrate the highest pass rate efficiency 95-100%. The rabbit mAb SP6 turned out to be less popular for the detection of Ki-67, less than 10% use among laboratories.(34,35)

Determination of the Ki-67 protein expression level plays an important role in differentiating breast cancer into molecular subtypes. Increased expression of the Ki-67 protein is associated with poor prognosis of breast cancer. Ki-67-positive cases are characterized by aggressive growth and metastasis. A high level of expression of the Ki-67 protein is associated with recurrence of breast cancer and overall low survival rate of patients. In normal breast tissue, the expression level of Ki-67 is very low.(36-38) Comparing the results of the expression of the biomarker Ki-67 of the breast cancer using ICC and IHC methods, a significant correlation was obtained. The concordance of the results of ICC and IHC studies ranged from 81 to 92%. (6,7,8,9) Therefore, developing the monoclonal antibody for Ki-67 protein with higher of binding specificity has great importance. Immunoreagents as the main components of test systems are being improved to increase the sensitivity, reliability and efficiency of use.

In the present study, we developing novel mAbs with high specificity and binding activity to Ki-67 protein. Specific binding with target molecule is a well-known characteristic of antibody. The high affinity and binding activity of antibodies provide specificity and reduction of false negatives. High affinity and specificity of 4B8 hybridoma strain mAbs (4B8 mAbs) to recombinant Ki-67 antigen was confirmed by indirect ELISA. The results Western blot showed that analytical sensitivity of the detection of Ki-67 antigen for 4B8 mAbs was below 100 ng. The application of newly generated anti-Ki67 mAbs in ICC provided showed the possibility of reliable detection of Ki-67 protein in cultured MCF-7 breast cancer cells. (39) The obtained 4B8 mAbs showed the identical level of nuclei staining as to the commercial anti-Ki-67 B56 mAbs that were used as a reference. B56 mAbs used as a positive control were obtained using a similar recombinant antigen Ki-67.(16) A large number of positively stained nuclei of cells and a bright intensity of their staining indicate a high level of proliferative activity of tumor cells in the studied preparations. Our results obtained from ELISA, Western blotting and ICC demonstrate that the newly developed mAbs are potentially useful as a immunodiagnostic tool for assessing the proliferative activity of breast tumor cells. The further research is focused on the use of obtained specific mAbs to evaluate the proliferative activity of breast tumor cells in FNA samples using ICC.

Conclusion

In this study, the synthesized the fragment of recombinant Ki-67 protein was used for obtaining of specific mAbs for assessing the proliferative activity of breast tumor cells. The production of specific mAbs, which makes it possible to reliably identify the Ki-67 biomarker protein, will make to use them for practical purposes - as a immunodiagnostic tool for the screening and early breast cancer diagnostics.

Acknowledgements

Authors Contribution

AS, KB, ES were involved in concepting and planning the research. All authors performed the data acquisition/collection, calculated the experimental data and performed the analysis, drafted the manuscript and designed the figures, aided in interpreting the results. All authors took parts in giving critical revision of the manuscript.

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