RESEARCH ARTICLE

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Generation and Characterization of Novel Diagnostic Mouse Monoclonal Antibodies Against Human Estrogen Receptor Alpha and Progesterone Receptor

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Abstract

Background: Estrogen and progesterone regulate the growth and development of several human cells and tissues. Their corresponding receptors (ER and PR) are important diagnostic and prognostic indicators for cancers of the breast and reproductive organs. Immunohistochemical analysis of ER and PR is the current standard method for evaluating the expression of these receptors in different cancers. Nonetheless, there is a significant lack of reproducibility of IHC results in laboratories worldwide, necessitating to develop more sensitive and specific antibodies for ER and PR IHC staining. **Methods:** ER and PR-specific monoclonal antibodies (MAbs) were generated by immunizing mice with synthetic peptides from ERα and PR. The isotypes and affinity constants of the selected MAbs were determined, and their specificities were assessed by peptide-specific ELISA, IHC, Western-blot analysis, and flow cytometry. In addition, the reactivity of generated MAbs was compared with that of the commercially-available anti-ER and anti-PR antibodies in IHC using normal and cancerous tissue sections. Moreover, 200 breast cancer tissue samples were stained using the newly generated MAbs along with commercial antibodies by IHC, and the sensitivity, specificity and accuracy of our MAbs were evaluated. **Results:** Among different MAbs generated in this study, two anti-ER and one anti-PR MAbs specifically detected the target antigens in normal and cancerous tissues in IHC. Further analyses confirmed the specificity of the MAbs in Western blotting and flow cytometry using a panel of ER and PR positive cell lines. The sensitivity, specificity and accuracy calculated for clone 1B9 (anti-ER) were 92.3%, 94.8% and 93%, and for clone 3D6 (anti-PR) were 93.0%, 94.3% and 93.5%, respectively. **Conclusion:** Our novel anti-ER and PR MAbs could be considered as suitable tools for diagnostic and research purposes.

Keywords: Estrogen receptor- progesterone receptor- monoclonal antibody- immunohistochemistry

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Introduction

Cancer is a significant public health problem worldwide. Estrogen and progesterone hormones affect many hormonal functions in different tissues (Ma and Yu, 2006). Estrogen and progesterone receptors (ER and PR) are primary regulators of the activity and development of different tissues, such as the uterus and mammary glands (Gao and Navaz, 2002). In addition to their role in the normal development of reproductive tissues, they are also involved in the development and progression of several cancers, such as breast, ovarian, testicular, and lung (Masi et al., 2021). Several studies have shown that by binding to their receptors in some tumors, such as tumors of the female reproductive system and breast, these hormones play a significant role in tumorigenesis and cancer cells growth (Cheng et al., 2018; Li et al., 2021; Roy and Vadlamudi, 2012). On the other hand, many reports are indicating that tumors expressing ERα and PR respond well to hormone-targeting therapies and chemotherapy and have a better prognosis and higher survival rates (El Sayed et al., 2019). The expression of ER is a good prognostic marker of clinical outcome in breast cancer and a useful predictive marker of response to adjuvant therapy (Louie and Sevigny, 2017). PR

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expression is also an independent valuable prognostic biomarker in breast cancer, and its expression level must be taken into account when deciding on adjuvant treatments in patients with hormone-receptor-positive early breast cancer (Bravaccini et al., 2020).

Considering these facts, the importance of checking ER and PR in tissues such as breast cancer is well documented, and determining the expression pattern of these receptors is currently essential to classify tumors and decide on therapeutic strategies (Dai et al., 2016).

Several methods exist for evaluating the expression pattern of hormone receptors. Among them, immunohistochemistry (IHC) has become the most common and preferred method for evaluating ER and PR expression in different cancers. This technique plays a vital role in the morphological evaluation of cancerous tissues and provides information regarding prognostic and predictive molecular markers for cancer treatment (Zaha, 2014).

Published studies have reported a significant rate of lack of reproducibility in ER and PR IHC results from different laboratories (De Jesus and Rosa, 2019; Griggs et al., 2017). Since ER and PR status is considered a validated predictive factor for anti-hormonal therapy of patients, generating sensitive and specific MAbs against these receptors for proper identification by immunohistochemical methods is essential. In this study, mouse MAbs against ERα and PR were generated and characterized, and their ability to detect the target receptors in cancer tissues was compared with commercially relevant MAbs.

Materials and Methods

Preparation of the immunogens

To induce immunity in mice against ERα and PR, different peptides were designed using Bcepred (https://webs.iiitd.edu.in/raghava/bcepred/index.html), COBEpro (http://scratch.proteomics.ics.uci.edu/), BepiPred (http://www.cbs.edu.dk/services/BepiPred/), and ABCpred (http://webs.iiid.edu.in/raghava/abc pred/index.html) websites. The peptides corresponding to aa 1-100 of ERα and aa 400-500 of PR were synthesized (ONTORES biotechnologies, Zhejiang, China) and conjugated to keyhole limpet hemocyanin (KLH: Sigma-Aldrich, Missouri, USA) carrier protein using sulfo m-maleimidobenzoyl-N-hydroxysulfosuccinimide Ester (Sulfo-MBS: Sigma Aldrich, USA) as a cross-linker. Accordingly, the linker and carrier were mixed in a ratio of 1:5 and incubated at room temperature for 2 hours on a shaker. Then, the solution was dialyzed against phosphate-buffered saline (PBS) to remove the unbound linkers. The conjugation quality was evaluated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

Hybridoma generation

Six-week female Balb/c mice (Animal Center of Pasteur Institute of Iran, Tehran, Iran) were immunized with each peptide. In the first injection, a mixture of 50 µg of each peptide was emulsified with an equal volume of complete Freund's adjuvant (CFA: Sigma Aldrich) and injected subcutaneously. Four weeks after the first injection, the mice were injected with 25 µg of the same peptide prepared in incomplete Freund's adjuvant (Sigma Aldrich) at 2-week intervals. Blood samples were collected from the mice before each injection, and the titers of the specific antibodies were determined using ELISA. The intravenous injection of 25 µg of each peptide diluted in PBS was performed after the titer of peptide-specific antibodies reached a plateau. After three days, the spleen cells of the mice were mixed with SP2/0 myeloma cells (NCBI 129, National Cell Bank of Iran, Pasteur Institute of Iran, Tehran) at a ratio of 5:1using 50% (v/v) PEG 1500 (Sigma Aldrich) as a fusing agent. The fused cells were cultured in Roswell Park Memorial Institute 1640 (RPMI 1640) medium (Sigma Aldrich) supplemented with 20% fetal calf serum, hypoxanthine, aminopterin, and thymidine, non-essential amino acids, and sodium pyruvate (all from Sigma Aldrich) as a selection medium.

Screening of hybridoma clones, purification, and identification of the MAbs

The supernatant of growing hybridomas was screened by an indirect ELISA. 96-well ELISA microplates (Corning, USA) were coated with five µg/mL ER and PR peptides in PBS for two h at 37 C. After washing with PBS containing 0.05% Tween20 (PBS-T) and one h blocking with 3% non-fat skimmed milk (Merck, Darmstadt, Germany), the plates were incubated with hybridoma cell supernatants for one h at 37°C. Next, the plates were washed, and HRP-conjugated sheep anti-mouse antibody (Sina Biotech, Tehran, Iran) was added and incubated for one h at 37 ° C. Finally, after washing the wells, tetramethylbenzidine (TMB) substrate solution (Pishtaz Teb Co, Tehran, Iran) was added to the wells. The reaction was stopped with 1N HCL, and optical densities (ODs) were measured at 450/620 nm using a multiscan ELISA reader (Biotek, Winooski, USA). In positive wells, the specific reactivity of the MAbs in the supernatants was checked by IHC using ER+/PR+ tissue sections. The selected hybridoma cells were sub-cloned four times by a limiting dilution assay (Loirat et al., 1992). Finally, 3-5×106 hybridomas were intraperitoneally injected to Balb/C mice pretreated with 500 ul pristane (Sigma Aldrich) to obtain ascites fluid rich in antibodies. Then, the ascites fluids were purified using a HiTrap Protein G column (GE Healthcare life sciences, USA).

Flow cytometry

To assess the reactivity of the MAbs with the native form of ER/PR, Hela cells were fixed with 4% paraformaldehyde (Sigma Aldrich) at room temperature for 20 minutes. The cells were then permeabilized with 0.01% saponin (Merck) for 15 minutes at room temperature and incubated with the anti-PR and anti-ER MAbs. After washing, the cells were incubated with FITC-conjugated sheep anti-mouse Ig PAb (Sina Biotech) for 45 min at 4˚C. An isotype-matched irrelevant MAb (2F9G5, Sina Biotech) was used as an isotype control. After washing, the cells were resuspended in PBS and were analyzed by a flow cytometer (Partec, Nuremberg, Germany) using Flow-jo 6.0 software.

Tissue specimens and IHC

A number of paired tissue sections, including tumor tissues of breast, endometrium, ovary, and lung and non-cancerous tissues of endometrium, cervix, and vagina, were collected. Also, 200 tissue sections of invasive ductal carcinoma of breast were tested by IHC. The slides were evaluated blindly by a pathologist and nuclear staining of ER and PR in ≥1% of invasive breast carcinoma tumor cells were considered positive according to the ASCO/CAP scoring guideline (Hammond et al., 2010). H-Scores were calculated based on the percentages of cells stained with the intensities 0 (none), 1+ (weak), 2+ (moderate), and 3+ (strong) giving a H-Score between 0 and 300 (Rizzardi et al., 2012). IBM SPSS statistics 26 was used for statistical analysis and calculation of specificity, sensitivity and accuracy of the MAbs. Normal and neoplastic human biopsy specimens were provided by the Pathology Department of Sina Hospital in Tehran. Briefly, tissue slides were deparaffinized in xylene (Sigma Aldrich) and rehydrated in graded ethanol (Merck) (100%, 90%, 70%, and 50%) and deionized water. Next, tissue sections were exposed to Tris-EDTA buffer in a 95 ° C water bath for heat-induced antigen retrieval. Endogenous peroxidase activity was blocked by immersing the tissue sections in 0.3% H2O2 for 15 minutes. After blocking with 5% sheep serum (SinaBiotech) for 30 minutes, the tissues were incubated with our MAbs, 1 µg/ml of 1B9 clone (anti-ER) and 3D6 clone (anti-PR), at room temperature for 1 hour. Commercial anti-ER (Clone SP1, Zytomed, Germany) and anti-PR (Clone.IHC751, GENOMEME, Canada) antibodies were used as the positive control. Negative control staining was performed with 1 µg/ml of normal mouse Ig. The samples were probed with a polymer detection system (BioVision, SinaBiotech) for 40 min. Diaminobenzidine (DAB) (SinaBiotech) was used as chromogen, and the samples were counterstained with hematoxylin (Padtanteb, Tehran, Iran), dehydrated, and mounted with Entellan (Merck).

Western blot analysis

MCF-7 cells were used to analyze the specific reactivity of MAbs with ER/PR in Western blot. MCF-7 cells were trypsinized and lysed using M-PER buffer (1ml/107 cells) containing protease inhibitors (Thermo Scientific Pierce, USA). After 10 minutes of incubation and centrifugation, total protein concentration was determined using a BCA Protein Assay Kit (Thermo Scientific). 40 µg of the cell lysates were run on SDS-polyacrylamide gel under reducing and non-reducing conditions and then were transferred to nitrocellulose membrane (Roche Diagnostics, Germany). The membranes were then blocked with 5% skimmed milk (prepared in PBS containing 0.05 % Tween 20 (PBST)) and incubated overnight at 4° C. After washing, the membranes were incubated for 45 minutes at room temperature with 1 µg/ml of the MAbs, followed by 45 min incubation with peroxidase-conjugated sheep anti-mouse Ig (Sinabiotech). Finally, enhanced chemiluminescence (ECL) substrate solution (GE healthcare life sciences) was used to visualize the blots.

Immunoglobulin isotype determination

Isotypes of the generated MAbs were determined using ELISA. Briefly, the generated MAbs and control antibodies were coated on the ELISA plate. After blocking, isotype-specific antibodies (goat anti-mouse IgG1, IgG2a, IgG2b, IgG3, IgA, and IgM antibodies, Sigma Aldrich) were added and incubated for 20 min at 37°C. After washing, HRP conjugated rabbit anti-goat antibodies (Sina Biotech) were added, followed by adding TMB. Then, the reaction was stopped, and ODs were measured at 450/620 nm.

Determination of antibody affinity constant

An ELISA-based method was used to determine the affinity constant of the produced MAbs (Kazemi et al., 2011). Briefly, three different concentrations of the estrogen receptor and progesterone receptor peptides, named [Ag], [Ag'], and [Ag''], were coated on ELISA plates. After 2 h of incubation at 37°C, the wells were blocked using 3% skimmed milk and incubated for 1 h at 37°C. Serial concentrations (2,000, 1,000, 500, 250. 125, 62.5, 31.25, 15.6, and 0 ng/ml) for anti-ER MAbs and (250. 125, 62.5, 31.25, 15.6, 7.8, 3.9 and 0 ng/ml) for anti-PR MAbs were added and incubated for one hour at 37° C. After washing, HRP-conjugated sheep anti-mouse Ig (Sina biotech) was added, followed by adding TMB substrate and stop solution, and ODs were measured at 450 nm. The sigmoidal curves were plotted using OD values of different antibody concentrations. To calculate the affinity constant, the antibody concentration of 50% of the maximum OD of each antigen concentration was calculated and placed in the formula "KD = 1/2(2 [Ab'] t - [Ab]t). [Ab']t and [Ab]t represents the antibody concentrations giving 50% of the maximum OD in two different concentrations of coated antigens where [Ag] = 2[Ag0]. The final affinity constant was obtained as the mean of the calculated values for the three different antigen concentrations.

Results

Monoclonal antibody production

Culture supernatants from fused hybridoma cells were screened. Several MAbs specifically reacting with ERα and PR were generated. In addition to ELISA, specific reactivity of the MAbs was assessed by IHC. Highly reactive clones, including two MAbs (2F2 and 1B9) against ERα and one MAb (3D6) against PR, were selected for the rest of the study. 1B9, 2F2 and 3D6 MAbs were affinity purified using protein G column and their concentrations after purification were 1.19 mg/ml, 0.81 mg/ml and 1.11 mg/ml, respectively.

Isotypes and affinity constants

All MAbs were purified from the ascitic fluid by protein G column, and their isotype and affinity were determined. The results are outlined in Table 1. The affinity constant of the MAbs was in the range of 2.3×10^{-9} to 5.3×10^{-10} M (Table 1). The affinity curves are shown in Figure 1.

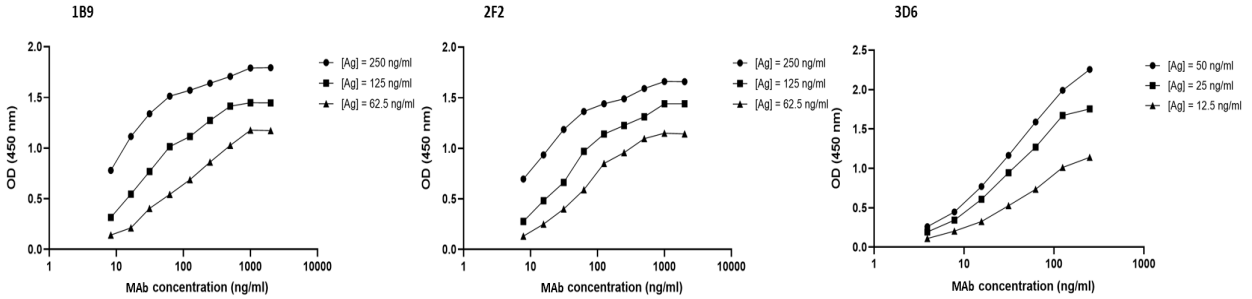

Figure 1. Standard Curves Obtained for the Calculation of the Affinity of the ER and PR Specific MAbs

Table 1. Isotypes and Affinity Constants of Generated MAbs against Human ERα and PR

Monoclonal antibody	Target antigen	Isotype	50% of Max OD for different antigen coatings			Affinity constant (M-1)
2F2	ERα	IgG1	250ng	125ng	62.5ng	5.32×10^{10}
			0.9	0.72	0.59	
1B9	ERα	IgG1	250ng	125ng	62.5ng	5.65×10^{10}
			0.88	0.77	0.57	
3D6	PR	IgG1	50ng	25ng	12.5ng	2.32×10^{9}
			1.12	0.88	0.57	

Immunoblotting analysis of anti-ERα and anti-PR MAbs

HeLa cell lysate was used to investigate the specific reactivity of anti-ERα and anti-PR MAbs with their cognate proteins. The results showed that 1B9 and 2F2 anti-ERα MAbs specifically recognized protein bands of 65 kDa corresponding to ERα in reduced and non-reduced conditions and did not show any cross-reactivity with other proteins (Figure 2(A)). 3D6, as a representative of anti-PR MAbs, was able to specifically react with reduced and non-reduced alpha and beta PR isotypes at 94 and 120 kDa (Figure 2(A)).

Flow cytometry profile of anti-ERα and anti-PR MAbs

To assess the specific reactivity of anti-ERα and

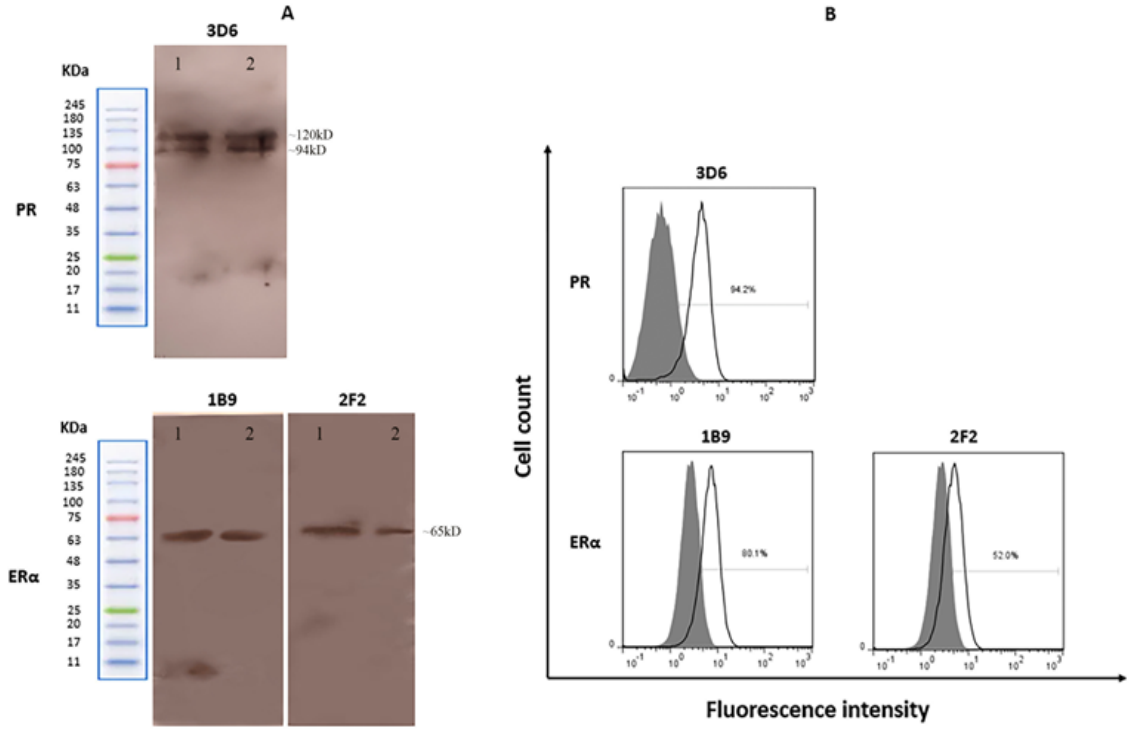

Figure 2. Western Blot and Flow Cytometry Analysis of anti-ERα and anti-PR Monoclonal Antibodies. A) Reactivity of anti-PR MAb (3D6) and anti-ERα MAbs (1B9 and 2F2) with lysate of HeLa cells were tested by Western blotting at reduced (lanes 1) and non-reduced (lanes 2) conditions. B) Reactivity patterns of anti-PR antibody (3D6) and anti-ERα monoclonal antibodies (1B9 and 2F2) with HeLa cells by intracellular staining and flow cytometry. The figures depicted for each antibody represent percent of positive cells as compared to cells stained with negative control MAb.

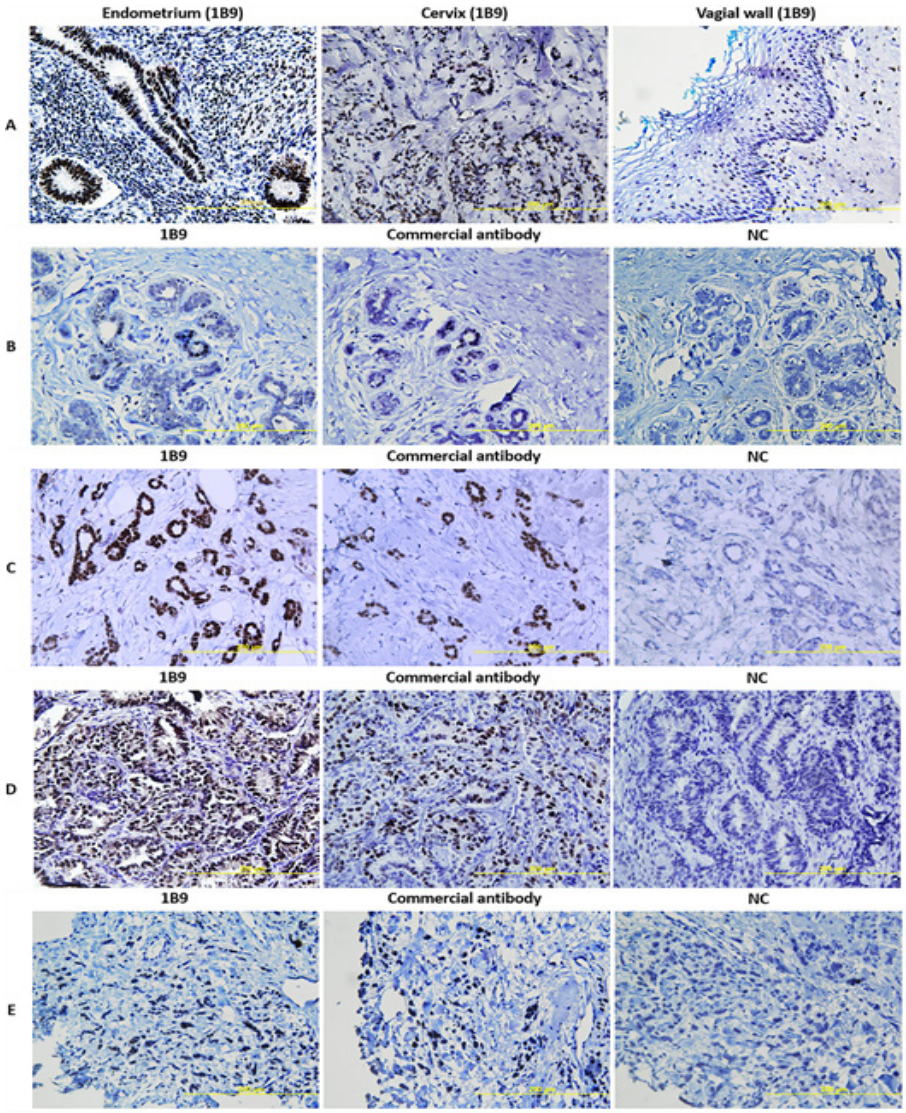

Figure 3. IHC Staining of Different Tissues with anti- ERα MAb (1B9). Reactivity of anti- ERα MAb 1B9 was tested by IHC in tissue sections from A) Normal endometrium, cervix, and vagina, B) Normal breast, C) Breast cancer, D) Endometrial cancer, E) Lung cancer; NC, Negative control

anti-PR with the native form of ERα and PR, we examined their binding to HeLa cells by intracellular flow cytometry staining. As shown in Figure 2 (B), anti-ERα MAbs (1B9, 2F2) and anti-PR MAb (3D6) recognized their corresponding native proteins in HeLa cells. 1B9 and to a lesser extent 2F2 recognized ER in 80.1% and 52% of HeLa cells, respectively. 3D6 recognized PR in 94.2% of HeLa cells (Figure 2 (B)).

IHC analysis of human normal and cancer tissues

Considering the significance of ER and PR detection in clinical diagnosis, the specific reactivity of generated MAbs was evaluated using IHC in breast and ovarian cancer tissue samples. As shown in Figure 3 and Figure 4, 1B9 and 3D6 specifically recognized their corresponding proteins with high specificity and sensitivity. The sensitivity of these antibodies to detect their target proteins was superior to that of commercial antibodies, as judged by the percentage of positive cells and strength of expression in tissue sections. No cross-reactivity with other proteins was detected (Figure 3).

Table 2. Sensitivity, Specificity and Accuracy of the ER and PR specific MAbs Calculated based on the Results of Staining of 200 Breast Cancer Tissue Samples

Target molecule	MAbs	Clone name	Number of the tested samples	Mean of H-Score* (SEM**)	Sensitivity	Specificity	Accuracy
ER	Our MAb	1B9	200	66.2(6.7)	92.30%	94.80%	93%
	Commercial MAb	SP1	200	65.3(6.1)	-	-	-
PR	Our MAb	3D6	200	24.1(4.1)	93.00%	94.30%	93.50%
	Commercial MAb	IHC651	200	24.0(4.1)	-	-	-

*Mean of H-Score: Mean of H-Score data obtained from IHC results of breast cancer patients; **SEM, Standard error of mean

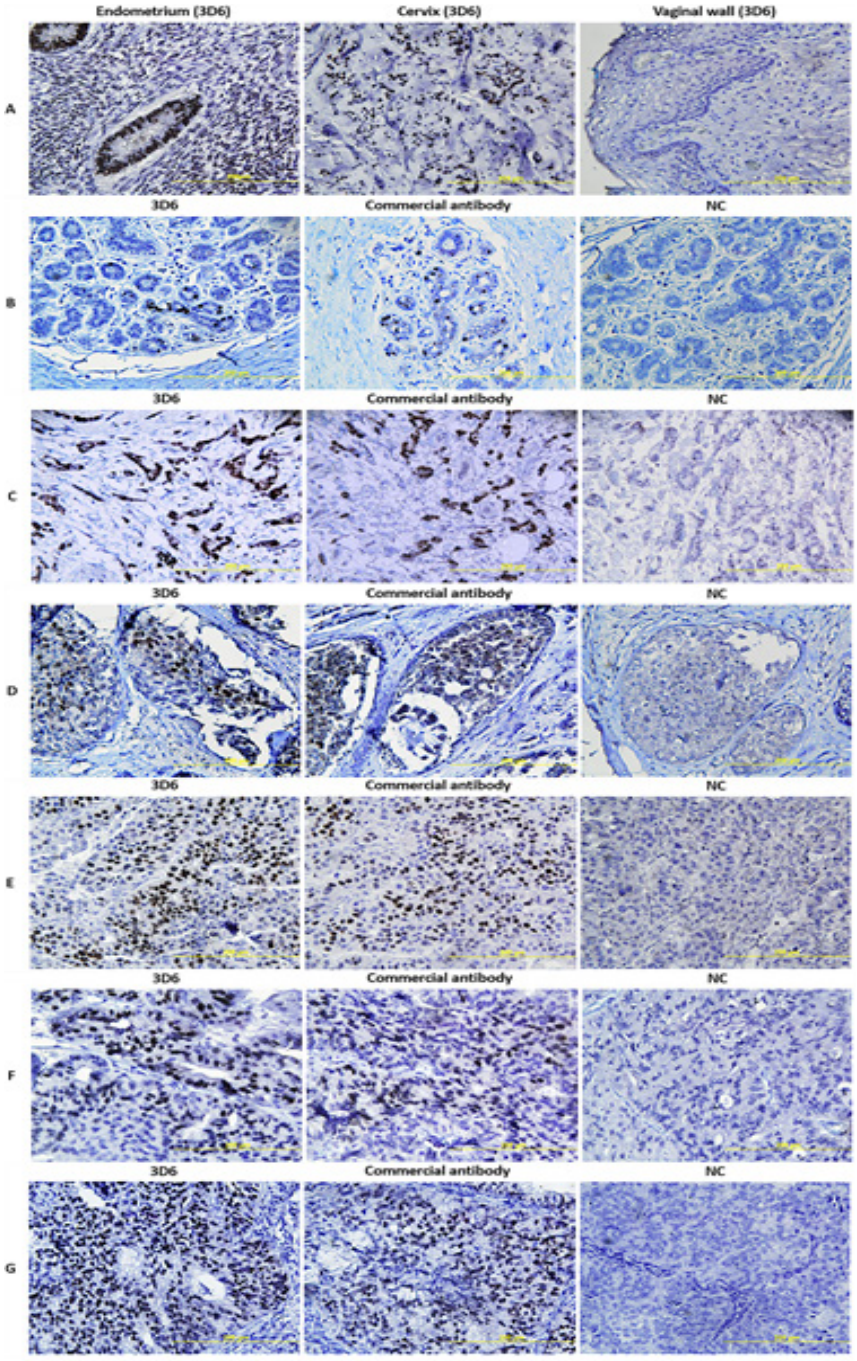

Figure 4. IHC Staining of Different Tissues with anti-PR MAb (3D6). Reactivity of anti-PR MAb 3D6 was tested by IHC in tissue sections from A) Normal endometrium, cervix, and vagina, B) Normal breast, C & D) Two different breast cancers, E & F) Two different ovarian cancers G) Endometrial cancer.

Evaluation of breast cancer samples by IHC test

Staining of 200 breast cancer tissue samples using the commercial and produced anti-ER and anti-PR by IHC test showed that the produced antibodies have the ability to accurately identify their target antigens. According to the results the mean H-Score of clones 1B9 and SP1 were 66.26 and 65.37, respectively, and these values were 24.18 and 24.01 for 3D6 and IHC651 clones, respectively. Based on IHC results, the sensitivity, specificity and accuracy of the produced antibodies in comparison with the commercial antibodies were also calculated and the results are presented in Table 2.

Discussion

Sex steroids and other growth factors play essential roles in breast epithelial tissue development, growth, and differentiation (Slepicka et al., 2021). ER and PR are expressed in more than 50% of breast cancer cases (Sohail et al., 2020). Their activation following ligand binding could trigger several gene networks and metabolic and regulatory pathways necessary to sustain cancer cell growth (Yaşar et al., 2016; Skildum et al., 2005). Therefore, ER and PR play critical roles in breast cancer development and progression; consequently, these receptors could be considered as useful diagnostic and

prognostic biomarkers in breast cancer (Feng et al., 2018). ER expression is a predictor of hormone therapy response, and it has also been shown to be a prognostic factor in breast cancer. Analysis of steroid receptor expression status has become a standard of care for breast cancer patients. ER expression level has been correlated with an enhanced likelihood of response to adjuvant therapy and prolonged disease-free survival. ER, and PR expression pattern is predictive of survival rate, treatment response, and prognosis (Zubair et al., 2021).

Among existing methods for evaluating the expression pattern of hormone receptors, IHC analysis is superior and showed higher discriminating power than biochemical assays for prognostic and predictive purposes (Duraiyan et al., 2012). However, variations in tissue preparation methods, types of antibodies, detection reagents, interpretation methods, and lack of technical standardization and inter-laboratory variations are among unsolved issues that might affect the predictability of IHC assay. Using antibodies with higher sensitivity, mainly when the hormone receptor expression level is low, is a critical factor affecting the result of IHC (Skildum et al., 2005). Therefore, the production of sensitive and specific MAbs against estrogen and progesterone receptors to detect low levels of these receptors is essential. Several anti-ER and anti-PR antibodies have so far been generated and characterized. For the first time, Greene (1980) developed three MAbs against human estrogen receptor using hybridoma technology with rat spleen-mouse myeloma fusion. Then they evaluated the reactivity of the antibodies in radioimmunoassay and immunocytochemistry assays but not IHC. Huang (2005) generated the first rabbit anti-estrogen receptor MAb, SP1, which was used in IHC on formalin-fixed and paraffin-embedded (FFPE) tissue sections. Their results showed that the affinity constant of SP1 was eight times higher than the standardized mouse monoclonal antibody to estrogen receptor (clone 1D5). The specificity and sensitivity of SP1 were similar to that of 1D5 in IHC of histologic specimens, indicating the great value of this newly developed anti-ER MAb in assessing ER status in human breast cancer.

Traish et al., (1990) produced murine monoclonal and rabbit polyclonal antibodies against PR using synthetic peptides corresponding to amino acids 533-547, 597-611, and 765-779 of the human progesterone receptor, which was conjugated to KLH carrier. Their results showed that only MAbs generated by the peptide amino acid 533-547 could recognize undenatured forms of PR. Generated MAbs specifically recognized PR, but not estrogen, glucocorticoid, or androgen receptors. In addition to recognizing PR from human breast cancer, the MAbs recognized this molecule from rabbit, mouse, calf, and rat uteri, indicating that the epitope recognized by these MAbs was conserved among these species. It is noteworthy that the reactivity of these MAbs was not evaluated in the IHC assay.

Huang et al., (2006) developed the first rabbit anti-PR MAb, SP2, which recognized its corresponding protein on FFPE breast cancer sections. SP2 recognized both isoforms of PR in Western blot (PR A and PR B), and the affinity constant of this MAb was 12 times higher than that of the commercial anti-PR MAb (1A6). Characterization studies showed that SP2 had important advantages over the currently available anti-PR antibodies, including reactivity even without heat-based antigen retrieval of fixed-embedded tissue sections in IHC assay. A comparative study of IHC on breast cancer histologic specimens showed that the reactivity of SP2 was similar to that of the commercial used anti-PR MAb 1A6, indicating the great value of this MAb for assessing PR expression in human breast carcinomas.

Kobayashi (2000) compared the reactivity of five commercial anti-ERα MAbs (purchased from different companies), which recognized different amino acid sequences, to determine their reliability in IHC staining. Their results showed that only one MAb, HC-20, was the most suitable for evaluating the ER expression status in human breast carcinoma sections in IHC. The rest of the MAbs did not give reliable results and could not be used for diagnostic purposes.

Another study conducted by Press et al., (2002) compared the specific reactivity of ten mice and two rabbit anti-PR MAbs for PR IHC immunostaining using the multi-tumor tissue sections. They realized that only two MAbs, PgR636 and PgR1294, stained the highest percentages of breast cancers with the highest concordance with the biochemical assay. Antigen retrieval was not necessary for immunostaining by these two MAbs in most tissues examined, although the reactivity of most other tested MAbs was highly dependent on antigen retrieval.

As mentioned above, several studies have so far generated and compared the reactivity of different anti-ER and anti-PR MAbs in terms of specificity and sensitivity with those of the commercial ones in the IHC assay. In the present study, we generated and characterized two anti-ER MAbs, 2F2 and 1B9, and one anti-PR MAb 3D6. Reactivity of our MAbs with PR and ER was evaluated by ELISA, flow cytometry, Western blotting, and IHC. All MAbs were able to recognize their corresponding target molecules in native form, as tested in flow cytometry. In addition, the MAbs were also able to detect ER and PR in denatured form, as they recognized these proteins in the lysate of HeLa cells checked by Western blotting. It is noteworthy that 3D6 recognized both isoforms of PR (PRα and PRβ). Our novel MAbs were evaluated by IHC in a large number of breast cancer tissue samples along with two diagnostic commercial MAbs, SP1 and IHC651 as standard MAbs. Clone SP1 is FDA cleared (Ross et al., 2019) and IHC651 is EU certified Mab (https://www.genomeme.ca/ihcaantibodies.html) which is currently being used as a diagnostic IHC tool in medical diagnostic laboratories around the world. The H-Score, sensitivity, specificity and accuracy of our MAbs in the IHC assay was found to be close to those of commercial antibodies, indicating their reliability to be used for determining the expression status of ER and PR in different human cancer sections which support their potential to be used in diagnostic tests.

In summary, highly specific and sensitive anti-ER and anti-PR murine MAbs were successfully generated and characterized in our study. The novel anti-ER and

anti-PR MAbs could detect their target receptors by IHC on formalin-fixed paraffin-embedded tissue sections with high sensitivity and specificity, making them a suitable tool for diagnostic application.

Author Contribution Statement

MHM performed the assays and prepared the original draft. MM, JKH, MAJ, TB and HAZ contributed to performing the experiments. HMT and ZM provided the tissue samples and evaluated the IHC results. FS, MMA, MJT, AHZ and FGSH reviewed and edited the manuscript. FS and MMA contributed to project conceptualization, data validation, project administration and supervision. All authors have read and agreed to the published version of the article.

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Ethical approval

All animal studies and human cancer tissues were conducted under the protocols approved by Tehran University of Medical Sciences. The Ethics Committee in Biomedical Research, Tehran University of Medical Sciences reviewed and approved this study (approval number IR.TUMS.SPH.REC.1400.011).

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Conflict of interest

The authors declare no conflict of interest.

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