Original Article

Iranian Journal of Immunology

https://iji.sums.ac.ir

Novel Monoclonal Antibodies Specific for Human Ki67 and P53 Tumor Markers in Breast Cancer Tissue Samples

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ABSTRACT

Background: Ki67 and P53 are important diagnostic and prognostic biomarkers expressed in several cancers. The current standard method for evaluating Ki67 and P53 in cancer tissues is immunohistochemistry (IHC), and having highly sensitive monoclonal antibodies against these biomarkers is necessary for an accurate diagnosis in the IHC test.

Objective: To generate and characterize novel monoclonal antibodies (mAbs) against human Ki67 and P53 antigens for IHC purposes.

Methods: Ki67 and P53-specific mAbs were produced by the hybridoma method and screened by enzyme-linked immunosorbent assay (ELISA) and IHC techniques. Selected mAbs were characterized using Western blot and flow cytometry, and their affinities and isotypes were determined by ELISA. Moreover, using the IHC technique in 200 breast cancer tissue samples, we assessed the specificity, sensitivity, and accuracy of the produced mAbs.

Results: Two anti-Ki67 (2C2 and 2H1) and three anti-P53 mAbs (2A6, 2G4, and 1G10) showed strong reactivity to their target antigens in IHC. The selected mAbs were also able to recognize their targets by flow cytometry as well as Western blotting using human tumor cell lines expressing these antigens. The specificity, sensitivity, and accuracy calculated for clone 2H1 were 94.2%, 99.0%, and 96.6%, and for clone 2A6 were 97.3%, 98.1%, and 97.5%, respectively. Using these two monoclonal antibodies, we found a significant correlation between Ki67 and P53 overexpression and lymph node metastasis in patients with breast cancer.

Conclusion: The present study showed that the novel anti-Ki67 and anti-P53 mAbs could recognize their respective antigens with high specificity and sensitivity and therefore can be used in prognostic studies.

Keywords: Immunohistochemistry; Ki67; Monoclonal Antibody; P53

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Cite this article as:
Hassanzadeh Makoui M, Mobini M, Khoshnoodi J, Bahadori T, Golsaz-Shirazi F, Moradi Tabriz H, Madjd Z, Jeddi-Tehrani M, Zarnani AH, Amiri MM, Shokri F. Novel Monoclonal Antibodies Specific for Human Ki67 and P53 Tumor Markers in Breast Cancer Tissue Samples. *Iran J Immunol.* 2023; 20(2):177-189,
doi: 10.22034/iji.2023.96926.2469.

Received: 2022-10-10
Revised: 2022-12-04
Accepted: 2022-12-08

INTRODUCTION

Globally, cancer ranks as a leading cause of death and its overall mortality is rapidly growing (1). The hallmark of cancer research is the discovery of different cancer biomarkers to facilitate the development of diagnostic and prognostic tools (2). At the same time, a major shift from non-specific cytotoxic drugs to specific monoclonal antibodies in cancer therapy has taken place (3).

Technological advances have contributed to the identification of various tumor biomarkers. These biomarkers can provide important information regarding predicting response to specific therapeutic interventions (as a predictive biomarker) and thus can be used for specific therapeutic regimens (4, 5). In addition, cancer biomarkers provide some information regarding the disease's outcomes such as cancer relapse and prognosis (6, 7). Several molecular markers, including Ki-67 and p53, are emerging tools for classifying, prognosis, and predicting treatment responses in many cancers (8).

The protein 53 (P53) was initially found in 1979 (9) and due to its considerable part in controlling cell division and thus arresting tumor formation and triggering a protective response, it is often termed the "Guardian of the Genome". As a tumor suppressor protein, wild-type P53 responds to DNA damage, oncogene activation, and hypoxia by orchestrating specific cellular responses including DNA repair pathways, apoptosis, and cell-cycle arrest (10).

P53 is expressed at low levels in normal cells of healthy individuals, while cancer cells often express its mutant forms. Researches show that the P53 gene is mutated in nearly 50% of cancers which leads to aberrant expression of the P53 protein (11). 18-25% of primary breast carcinomas contain mutations in P53. Regular expression and the lack of mutations in P53 are related to longer disease-free survival (DFS) after primary therapy in patients with breast cancer (12). In addition, overexpression of P53 has been noticed in the majority of human malignancies and its upregulation reported to be associated with tumor progression and poor prognosis (13).

Ki67 is a cell proliferation marker that is present in all cell cycle phases except G0 (14). This protein is present in proliferative cells and is overexpressed in cancer cells (15). As a proliferation marker, Ki67 is used for grading several kinds of cancers such as squamous cell carcinoma, breast cancer, and glioma (16-18). The evaluation of this marker in cancer patients can provide helpful information on the prognosis of the disease, the response to neoadjuvant therapy, and treatment outcome in different malignancies such as gastrointestinal, breast, and prostate cancers (19-21). Despite the production of several antibodies against P53 and Ki67 (22, 23), researchers are still trying to produce antibodies with high sensitivity and specificity that can be used in diagnostic or prognostic tests. Here, we report the production and characterization of a panel of mouse mAbs that specifically recognize human Ki67 and P53.

MATERIALS AND METHODS

Tissue Collection

Some matched tissue samples, including tumor tissues from the breast, prostate, gastric, ovarian, colorectal, bladder, pancreas, brain cancers, noncancerous tissues from the tonsil, breast, testis, and pancreas were collected. The Pathology Department of Sina Hospital (Tehran, Iran) provided normal and cancerous tissue samples.

Preparation of Immunogens

In this study, different synthetic peptides from the amino acid sequences of Ki67 and P53 molecules were designed and used for mAb generation. ABCpred (http://webs.iiid.edu.in/raghava/abcpred/index.html) Bcepred (https://webs.iiitd.edu.in/raghava/bcepred/index.html), BepiPred (http://www.cbs.edu.dk/services/BepiPred/), and COBEpro (http://

scratch.proteomics.ics.uci.edu/) websites were used to find suitable epitopes on target antigens and design peptides (24). Selected peptides, corresponding to the middle part and C-terminal regions of Ki67 (corresponding to aa1200-1300) and P53 (corresponding to aa 290-393), respectively, were synthesized (ONTORES biotechnologies, China) and coupled to KLH (Sigma–Aldrich, USA), as the carrier protein, using Sulfo-MBS (Sigma–Aldrich) cross-linker as described by Zarei et al. (25).

Hybridoma Generation

Three female Balb/C mice, for each target antigen, were purchased from the Pasteur Institute in Iran and subcutaneously immunized with 50 µg of KLH-conjugated peptide combined with complete Freund's adjuvant (Sigma–Aldrich). After 4 weeks, 5 subsequent boosters were given biweekly with 25 µg of the antigen emulsified in incomplete Freund's adjuvant (Sigma–Aldrich). After each immunization, the blood samples were taken for evaluation of antigen-specific antibody response. After confirming that the antigen-specific antibody titers had reached a plateau, the mice were challenged intravenously with 25 µg of conjugated peptides 3 days before fusion. Splenic cells were isolated from the individual mice and fused with murine SP2/0 myeloma cells (National Cell Bank of Pasteur Institute, Tehran, Iran) as previously described (26).

Screening of Hybridomas and Purification of Specific Monoclonal Antibodies

After hybridoma cells were selected in a culture medium containing HAT, their supernatants were screened for the presence of specific antibodies by an indirect ELISA. Peptides were coated at a concentration of 5 µg/ml in microtiter polystyrene plates (MaxiSorp, Nunc, Glostrup, Denmark). After blocking with 3% skim milk (Merck, Germany), cell supernatants were added to the plates for 1 h followed by the addition of appropriate dilution of sheep anti-mouse antibody conjugated with HRP (Sina Biotech Co., Tehran, Iran) for 1 h. After washing them, TMB substrate solution (Sina Biotech Co.) was added. Finally, the reactions were stopped by adding 1M HCl stop solution, and the optical density (OD) was measured by an ELISA reader (Biotek, Winooski, USA) at 450/620 nm. Wells with good reactivity in ELISA were screened by the IHC staining using the P53-positive breast cancer and human normal tonsil tissue sections, which naturally express Ki67.

Individual Balb/C mice were intraperitoneally injected with 500 µl pristine (Sigma-Aldrich) to get ascites fluid that contains a high concentration of mAbs. Seven days later, the mice were intraperitoneally injected with 3-5×10^6 hybridoma cells (in 500 ul PBS). Seven to ten days after the injection, the produced ascites were collected and the antibodies were purified by protein G affinity columns (GE Healthcare, Chicago, USA).

Flow Cytometry

Indirect intracellular staining was conducted to detect Ki67 and P53 in HeLa cells and MDA-MB-231 cells, respectively. The cells were harvested and washed with PBS containing 2% FBS and fixed as previously explained (26). After permeabilization with 0.01% Saponin (Merck) (15 min at 25 °C), the cells were incubated with 2-4µg/ml of relevant or isotype-matched irrelevant (2F9G5) mAbs for 1 h at 4 °C. After washing them three times, the sheep anti-mouse antibody conjugated with FITC (Sina Biotech) was added to the cells and incubated at 4 °C for 45 min. Finally, the cells were washed and suspended in PBS and analyzed using a flow cytometer (Partec, Nuremberg, Germany), and the data was analyzed employing Flowjo 6.0 software.

Tissue Specimens and IHC

3-µm sections of formalin-fixed, paraffin-embedded normal and cancerous tissues were used in this study. We tested 200 tissue specimens of invasive ductal carcinoma of

the breast using IHC. A pathologist blindly assessed the IHC slides. In addition, tumor tissues with ≥20% of Ki67 positive cells were considered highly proliferative, and tumors with <20% of Ki67 positive cancer cells were considered low proliferative (27). P53 was scored as follows: negative (focal staining, <10% of cancer cells), 1+(heterogeneous or focal staining, 10 to 49% of cancer cells), and 2+(homogeneous and diffuse staining, ≥50% of cancer cells) (28). The percentages of stained cells and their staining intensities 3+(strong), 2+(moderate), 1+(weak), and 0 (none) were used to calculate the H-Score values (a score between 0 and 300). Statistical analysis was done using IBM SPSS statistics 26 software. The specificity, sensitivity, and accuracy values for 2H1 and 2A6 clones were calculated using MedCalc online software (29). Briefly, tissue sections were deparaffinized in xylene, and rehydrated in graded ethanol (100, 90, 70, and 50%), and deionized water. Then, the heat-induced antigen retrieval was carried out using Tris-EDTA buffer (PH:9) in a water bath (at 95 °C for 30 min). The sections were treated for 15 min with 0.3% hydrogen peroxide solution to block endogenous peroxidase activity. After blocking with 5% goat serum for 30 min, the sections were incubated with pre-optimized concentrations of the selected mAbs 2H1 (Ki67) and 2A6 (P53), (1.5 and 1 µg/ml, respectively) at 25 °C for 1 h. In the negative control slides, mAbs were substituted with an equivalent concentration of normal mouse immunoglobulin. Mouse anti-human Ki67 mAb (clone. IHC067, GENOMEME, British Columbia, Canada), rabbit anti-human Ki67 mAb (clone.SP6, Master Diagnostica, Granada, Spain), and mouse anti-human P53 mAb (clone.IHC053, GENOMEME) mAbs were used as the positive controls. The slides were then incubated with a polymer detection system (Sina Biotech Co.) for 40 min and subsequently with diaminobenzidine (DAB) (Sina Biotech). Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted with Entellan (Merck).

Western Blot Analysis

The specificities of mAbs were analyzed by the Western blot assay. HeLa and MDA-MB-231 cells (10^7 cells/ml) were harvested and lysed in M-PER buffer with protease inhibitors (Pierce, Rockford, IL). BCA Protein Assay Kit (Thermo Scientific) was utilized to determine the total protein concentration. Cell lysates were denatured by boiling for 5 min in the presence or absence of 2-ME (Non-reduced and reduced conditions, respectively). Cell lysate samples were loaded for gel electrophoresis at 20 µg/sample under non-reducing and reducing conditions. After separation, the proteins were transferred onto nitrocellulose membranes (Roche Diagnostics, Mannheim, Germany). Membranes were blocked with PBS-Tween 20 containing 5% non-fat skim milk overnight at 4 °C, and after washing with 5% skim milk containing 0.05% Tween 20, the membranes were incubated with the corresponding mAbs at 37 °C for 45 min. All the primary antibodies were used at a concentration of 1µg/ml. After another washing, the membranes were incubated at room temperature for 45 min with sheep anti-mouse Ig conjugated with HRP (Sina Biotech) at a dilution of 1:1500. Enhanced chemiluminescence (ECL) substrate solution (GE Healthcare) was used for visualizing the bands and finally, the bands were exposed to x-ray films.

Isotyping Selected mAbs by ELISA

A commercial ELISA kit (Sigma-Aldrich) was conducted to discover the class and subclass of the generated mAbs. In brief, ELISA plates were coated with purified mAbs, and after washing and blocking, the isotyping antibodies were added. After adding rabbit anti-goat antibodies (Sina Biotech) and then TMB substrate solution, the reactions were stopped with a stop solution, and the OD values were determined as described earlier.

Affinity Constant Determination

Affinity binding constants of generated

mAbs were determined by an ELISA-based method. Briefly, three different concentrations of the target peptides were coated on an ELISA plate and assigned [Ag], [Ag'], and [Ag''], respectively. After blocking, serially diluted mAbs (500, 250. 125, 62.5, 31.25, 15.6, and 0 ng/ml) were added. After 1 h of incubation, sheep anti-mouse Ig-peroxidase conjugate (Sina Biotech) was added. The ODs were measured at 450 nm, and the curves were constructed (30). Next, we used the obtained ODs against the logarithmic concentrations of antibodies to construct sigmoidal curves. In the end, the antibody concentration giving 50% of the maximum absorbance value ([Ab] t) was utilized to calculate the K_D of the mAbs using the formula K_D=1/2(2 [Ab']t - [Ab]t). [Ab']t and [Ab]t represent the concentrations of mAbs resulting in 50% of the maximum OD at two different concentrations of coated antigens where [Ag]=2[Ag0]. The mean of the three affinity constants calculated for the three different antigen concentrations was considered the final K_D value.

Evaluation of the Association between the Investigated Biomarkers and Tumor Characteristics

The correlation between the Ki67 and P53 tumor markers and tumor demographic characteristics was investigated in 200 tissue samples of breast cancer patients using the Spearman and/or the Pearson's X2 tests. IBM SPSS statistics 26 software was used to perform statistical analysis.

Ethical Statement

This study is conducted in accordance with the guidelines from the Tehran University of Medical Sciences for laboratory animals and human specimens under the ethical permits IR.TUMS.SPH.REC.1400.011 and approved by the Ethics Committee in Biomedical Research of Tehran University of Medical Sciences.

RESULTS

Development of Monoclonal Antibody

After cell fusion and screening of culture supernatants by ELISA, 30 clones producing high titers of specific mAbs against P53 and 30 clones against Ki67 were established. The reactivity of the mAbs with tissue samples expressing their corresponding antigens was further tested by IHC, and the clones with the highest reactivity in IHC have been chosen for the remaining tests. Two mAbs designated 2H1 and 2C2 against Ki67 and three mAbs designated 1G10, 2G4, and 2A6 against P53 showed the highest reactivity in the IHC test and were expanded for ascites production. The isotype and affinity constant of the purified mAbs are shown in Table 1.

Anti-Ki67 and -P53 mAbs Specifically Recognize Ki67 and P53 in Cell Lysate

The specific reactivity of the selected mAbs with their corresponding antigens under reducing and non-reducing conditions were determined by Western blotting. Anti-Ki67 and anti-P53 mAbs specifically recognized the single bands of 319 and 53 KD proteins of human Ki67 and P53 in the cell lysates, respectively, under both reducing and non-reducing conditions (Figure 1A).

Table 1. Affinity constant and isotype of selected mAbs against human Ki67 and P53

Monoclonal antibody	Target antigen	Isotype	Affinity constant (M^{-1})
2H1	Ki67	IgG1	2.30×10^9
2C2	Ki67	IgG1	1.56×10^9
2A6	P53	IgG1	3.24×10^9
2G4	P53	IgG1	2.23×10^9
1G10	P53	IgG1	1.61×10^9

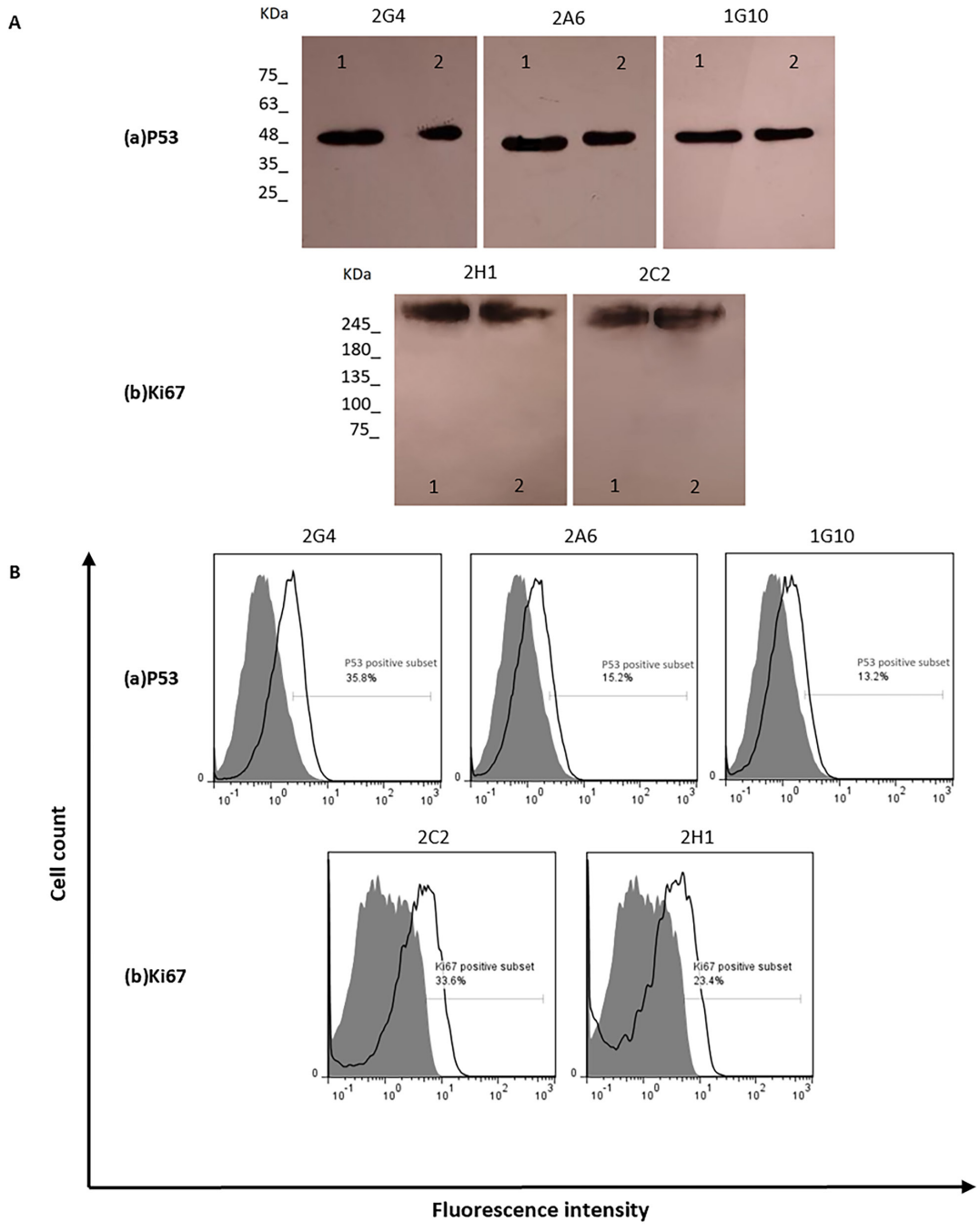

Figure 1. Flow cytometry and western blot analysis of anti-P53 and anti-Ki67 monoclonal antibodies. A) Western blot profiles of anti-P53 mAbs (clones: 2G4, 2A6 and 1G10) with lysates of MDA-MB-231 cell-line (a) and anti-Ki67 mAbs (clones: 2H1 and 2C2) with lysates of HeLa cell-line (b) at reduced (lane 1) and non-reduced (lane 2) conditions. B) Flow cytometry profiles of (a) anti-P53 mAbs (2G4, 2A6 and IG10) using MDA-MB-231 cell-line and (b) anti-Ki67 mAbs (2C2 and 2H1) using HeLa cell-line.

Anti-Ki67 and Anti-P53 mAbs Specifically Recognize Ki67 and P53 in a Native Form

The ability of anti-P53 and anti-Ki67 monoclonal antibodies to recognize their corresponding antigens in a native form on P53- (MDA-MB-231) or Ki67- (HeLa) expressing cell lines was assessed by flow cytometry. 2G4 (58.9%) and to a lesser extent 2A6 (32.9%) and 1G10 (30.6%) recognized P53 in MDA-MB-231 cells. 2H1 and 2C2 were able to recognize Ki67 in HeLa cells at almost similar levels (35% and 47.4%, respectively) (Figure 1B).

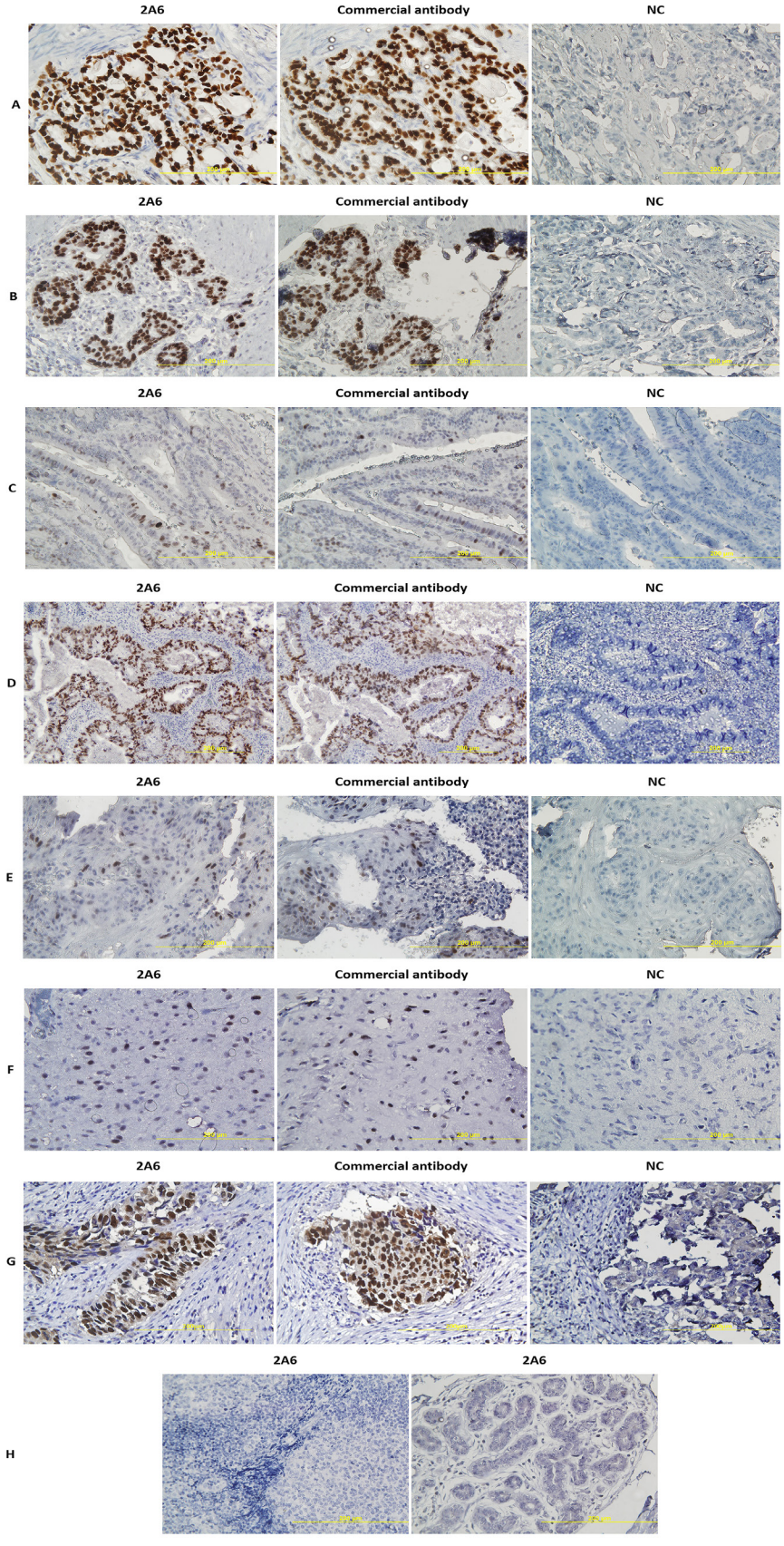

Figure 2. IHC staining of different tissues with anti-P53 mAb (2A6). Reactivity of anti-P53 mAb 2A6 was tested by IHC in cancer and normal tissues from different origins. A-C: Gastric cancer, D: Bladder cancer. E: Oral mass, F: Glioblastoma, G: Breast cancer, H: (a) human tonsil and (b) normal breast. NC: Negative control

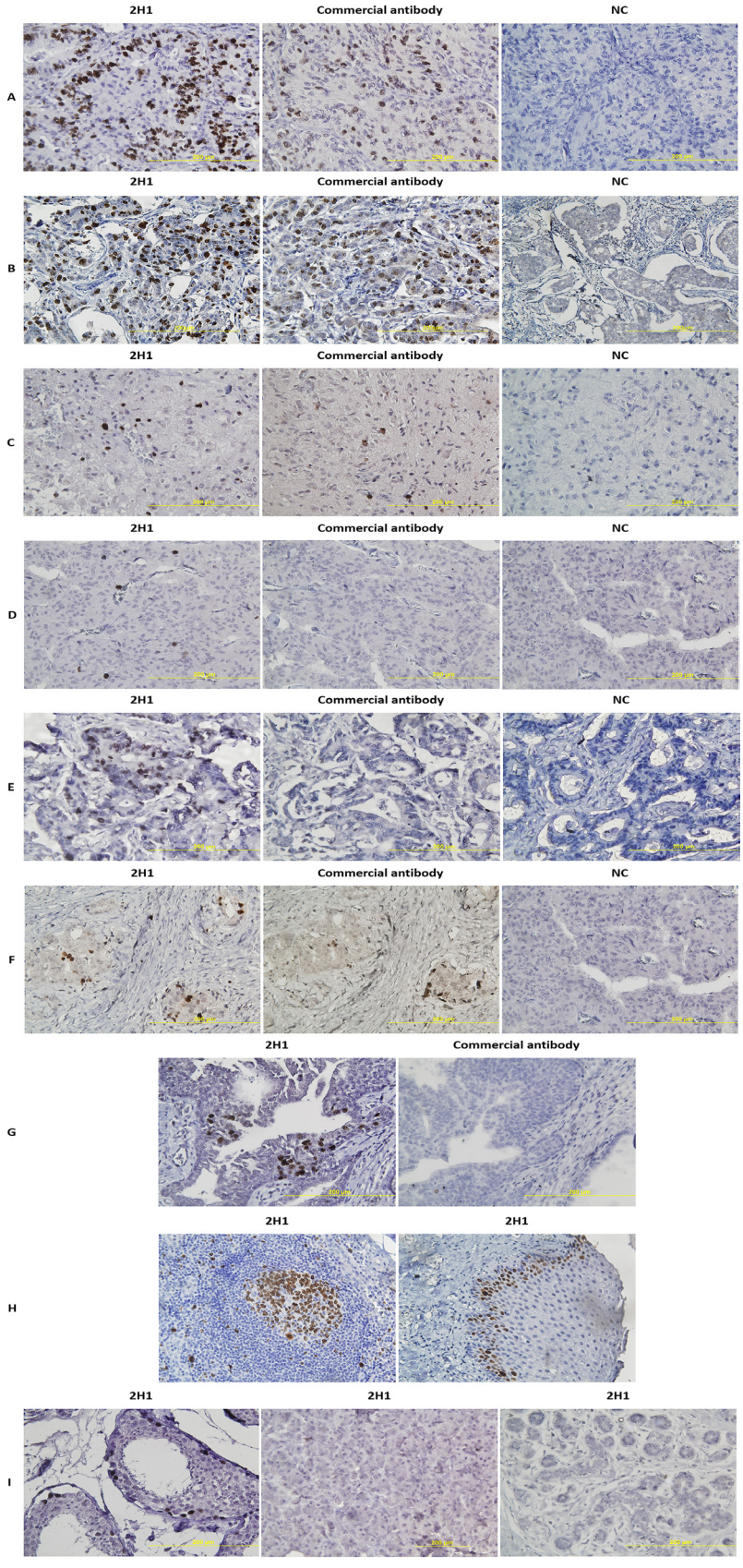

Figure 3. IHC staining of different tissues with anti-Ki67 mAb (2H1). Reactivity of anti-Ki67 mAb 2H1 was tested by IHC in cancer and normal tissues from different origins. A) Ovarian cancer, B) Breast cancer, C) Glioblastoma, D) Pancreas cancer; E) Colorectal cancer; F-G) Prostate cancer; H) Human tonsil, I) From left to right, normal testis, normal pancreas and normal breast. NC: Negative control

Anti-Ki67 and Anti-P53 mAbs Showed a Specific Pattern of Reactivity in the IHC Staining of Different Cancer Tissues

The IHC staining was carried out to evaluate the prognostic potential of the anti-P53 and anti-Ki67 mAbs. Commercial mAbs were used as the positive controls. Based on the preliminary IHC results, clones 2A6 and 2H1 showed superior reactivity compared with the other clones and therefore these clones were selected for the IHC staining (data not shown). Our results revealed that both selected mAbs were able to specifically react with their target nuclear antigens in different cancer tissues, including gastric, bladder, oral, brain, breast, ovarian, pancreas, colorectal, and prostate cancers (Figures 2 and 3). No background staining was observed. In addition, the negative control slides showed no reactivity. A comparison between these mAbs and the commercial counterparts showed that in some tissues especially those with low antigen expression, the intensity of staining with our mAb 2H1 was substantially stronger than that of the commercial control (Figures 3A-G). 2A6 displayed a similar pattern of reactivity to that of the commercial mAb (Figure 2).

Assessment of Breast Cancer Tissue Sections Using the IHC Test

The results regarding the IHC tests of 200 breast cancer tissue samples that were stained by commercial and produced anti-Ki67 and anti-P53 revealed that the newly generated mAbs can accurately detect their target antigens. Based on the results, the mean H scores of clones 2H1 and SP6 were respectively 12.2 and 12.7, and these values were 22.4 and 22.3 for 2A6 and the IHC651 clones respectively. The specificity, and accuracy of the generated mAbs in comparison with commercial ones were evaluated based on the IHC findings and the relevant results are shown in Table 2. Also, the expression level of Ki67 and p53 was investigated in breast cancer patients and the association of these biomarkers with different tumor characteristics, including tumor type, stage, grade, size as well as tumor metastasis were evaluated. A significant correlation was observed between the expression levels of Ki67 and P53 (P=0.001, r=0.298). Furthermore, a significant correlation was found between Ki67 overexpression and the tumor's lymph node invasion (P=0.039, r=0.206). However, no significant correlation was detected between the expression levels of these two biomarkers and other demographic characteristics of the tumors.

DISCUSSION

High-affinity mAbs against Ki67 and P53 are necessary elements for the specific and sensitive detection of these proteins in cancer tissues. The expression profile of these two proteins has several clinical applications such as tumor screening and diagnosis confirmation, monitoring of therapeutic responses, disease prognosis, and the prediction of tumor recurrence (31, 32). Therefore, the development of new mAbs for more sensitive and specific detection of these

Table 2. H-Score means, specificity, sensitivity and accuracy results of staining of 200 breast cancer tissue samples using the produced and commercial antibodies; confidence interval of the mean: 95%

Target antigen	Tested mAb	Clone name	Number of samples	Mean of H-Scores*	SD**	Sensitivity	Specificity	Accuracy
Ki67	Our mAb	2H1	200	12.2	22.4	99.0%	94.2%	96.6%
	Standard mAb	SP6	200	12.7	27.6	-	-	-
P53	Our mAb	2A6	200	22.4	58.3	98.1%	97.3%	97.5%
	Standard mAb	IHC053	200	22.3	58.9	-	-	-

*Mean of H-Score: Mean of H-Score data collected from the IHC results of breast cancer patients; **SD: Standard deviation

biomarkers is crucial.

In the present study, we designed and employed specific peptides to generate highly specific mAbs against Ki67 and P53 tumor markers to be used in the IHC assay. Since tissue fixation with formalin and antigen retrieval during IHC affects the conformation of many epitopes located in the target molecule, antibodies reacting with linear epitopes are preferred. In this regard, we designed and used peptides for mice immunization and mAbs production. Then, we characterized these mAbs and investigated and compared their reactivity and specificity with the commercially available mAbs using various normal and cancerous tissues.

Among the 30 clones producing specific mAbs against P53 or Ki67, two anti-Ki67 mAbs 2H1 and 2C2 and three anti-P53 mAbs 1G10, 2G4, and 2A6 showed the highest reactivity and lowest background in IHC and were selected for further characterization. Our results showed that similar to the standard commercial clones, our anti-Ki67 mAb 2H1could detect low expression levels of Ki67 at high sensitivity and displayed a specific reactivity with nuclear antigen and low non-specific binding in different tissues tested (Figure 3). Differences in reactivity pattern of anti-Ki67 antibodies has also been reported earlier. In one study (33), the reactivity of two frequently used anti-Ki67 mAbs (MIB-1 and MM1) with two polyclonal anti-Ki67 antibodies (Rah Ki-67 and NCL-Ki-67p) was compared by the IHC test. According to the results, MIB-1 exhibited better visual staining compared with the other antibodies. One possible explanation for such differences could be the different epitopes that the antibodies react with. Some linear epitopes might be more susceptible to conformational changes induced by protein denaturing parameters such as formalin which may result in epitope instability or the weakness of antibody binding to the target epitope. Antibody affinity to the corresponding epitope also plays a crucial role in the sensitivity and specificity of the mAb. Ki-67 has two protein-coding forms with different expression patterns (34), which may be a source of variation in the antibody reactivity. Moreover, mAbs are expected to display less non-specific binding than polyclonal ones in the IHC test. Nonetheless, the judgment of the overall reactivity of different antibodies needs a comprehensive survey using a panel of different cancerous and normal tissues expressing the target antigen at varying levels. On the contrary, Owens et al. (35) showed that the total number of positive cells after staining with MIB-1 and MM1 mAbs was equal.

Next, we compared the reactivity pattern of anti-P53 mAb 2A6 and a commercial mAb IHC053 using a panel of cancer and normal tissues expressing P53. Both antibodies displayed comparable reactivity patterns and were able to detect P53 even at low expression levels with high specificity (Figure 2).

To validate the specificity of our mAbs, we tested their reactivity with cognate antigen in the lysate of MDA-MB-231 and Hela cells by Western blot technique. All selected clones showed a single band with predicted molecular weight (Ki67: 359 KDa and P53: 53KDa) indicating their specific reactivity. Turpeinen et al. (36) compared the reactivity of six known anti-P53 antibodies with the lysate of mouse skin cells and MCF7 cells in Western blotting. Their results showed that the two polyclonal antibodies (CM1 and CMS) were not suitable for Western blotting due to the presence of antibody impurities after protein G purification. Among the other four mAbs, only DO7 could detect P53 without a nonspecific band. One of the reasons for the presence of additional bands in their Western blot results could be the cross-reactivity of the anti-P53 antibodies with P63 and P73, which have structural homology and similar epitopes. These results were subsequently confirmed by Bonsing et al. (37), who showed that most of the tested anti-P53 antibodies recognize antigens other than P53 in Western blotting. However, as shown in part A of Figure 1, the clones produced

in this study recognize a single band with a molecular weight of approximately 53 KDa. These findings highlight the importance of the anti-P53 mAbs produced in our study which specifically recognizes P53 by IHC and Western blotting.

We also assessed the reactivity of our P53 and Ki63-specific mAbs by flow cytometry. All the mAbs were capable of binding to their target molecules expressed on the selected tumor cell lines. The level of reactivity was generally weak to moderate. This might be due to the intracytoplasmic staining which is required to detect nuclear antigens and also the fact that these mAbs were raised against selected peptides and primarily recognize linear epitopes. Indeed, many of the mAbs generated against these two molecules have so far been reported (38, 39), and some of those available through commercial companies (Clone 261352 of Novus Biologicals, MAB18391 of R & D Systems, 278570 and 278730 of ABCAM, pS37 of BD Biosciences for anti-P53 antibodies; ab279653 of ABCAM, 1297A of Novus Biologicals and 350511 of Biolegend for anti-Ki67 antibodies) display a similar pattern of reactivity. It has been reported that p53 expression may correlate with both grade and ploidy of breast cancer, and its detection by flow cytometry may be of clinical importance (40). Our mAbs' specificity, sensitivity, and accuracy in the IHC test were close to that of the commercial antibodies, demonstrating their reliability to be used for discovering the expression status of Ki67 and P53 in various human tumor sections and the high specificity and sensitivity of these mAbs support their capacity for use in prognostic studies.

Our data showed a significant correlation between overexpression of Ki67 and lymph node invasion which is consistent with the results of other studies (41-43). We also observed a significant correlation for the co-expression of both P53 and Ki67 biomarkers which is compatible with the results reported by Allred et al. (44). These results further prove the importance of examining Ki67 and P53 in breast cancer as prognostic factors.

In summary, we successfully generated novel anti-Ki67 and anti-P53 mAbs that specifically recognize these tumor biomarkers in human cancer tissues, making them a valuable tool for prognostic studies.

ACKNOWLEDGMENT

The authors would like to take this opportunity to thank the personnel of Sina Hospital's Pathology Department affiliated with the Tehran University of Medical Sciences for providing tissue samples. This study was partially supported by the Tehran University of Medical Sciences (Grants No. 99-1-99-46975 and 99-1-99-47173).

Conflict of Interest: None declared.

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