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Investigating monoclonal antibody against cytokeratin 19 tumor marker

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Abstract

Cytokeratin 19 (CK 19) is a member of the intermediate filaments that are widely expressed in thyroid, breast, colon, small and non-small cell lung, and prostate cancer cells. This paper aims to produce the monoclonal antibody against CK 19. Recombinant CK 19 was used to immunize of two 6–8 weeks old female Balb/c mice. For the first injection, recombinant antigen was mixed with the complete Freund's Adjuvant, and for the second and third injections, mixed with the Incomplete Freund's Adjuvant. Injections were performed at intervals of 15 days. Four days after the third injection, titration of mice serum was determined using indirect ELISA and better-immunized mice selected for fusion. Intravenous (IV) injection was performed 4 days before the fusion. The spleen cells of the mice were fused with sp2/0 cells using 50% v/v polyethylene glycol (PEG) and cells were suspended in HAT (Hypoxanthine-Aminopterin-Thymidine) medium. Supernatants of hybridoma cells were screened for antibody secretion by indirect ELISA. Two stable hybridomas were obtained among 78 hybridoma clones that reacted with the recombinant CK 19 by indirect ELISA. These hybridoma cells were monoclonal twice by limiting dilution. Besides, immunization by recombinant CK 19 led to the obtaining of two stable monoclonal antibodies against this antigen. Western blotting with HT-29 cell line demonstrated a band of upper than 43 kDa. Result of HepG2 indicated a band of lower than 45 kDa while MCF7 bands of 45 kDa and higher were observed for MCF7. A431 cell line was epidermoid carcinoma, as CK 19 did not exist in the epidermis, therefore no band was observed in A431 cell line.

Keywords CK19, Monoclonal antibody, Tumor marker

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Introduction

Cytokeratin is a member of intermediate filament proteins which is expressed in epithelial cells and is divided into two groups: acidic cytokeratins (cytokeratin 9–20) and basic cytokeratins (cytokeratin 1–8) [1]. Normal epithelial cells express at least one type of acidic cytokeratin and one type of basic cytokeratin. Cytokeratins play various roles such as maintaining the integrity of cell, signaling, and apoptosis [2]. Clinically, cytokeratins are important tumor markers in diagnosis and follow-up of patients with various cancers. Cytokeratins reflect tumor cell activity, therefore following-up of cancer patients with repeated tests of cytokeratin; as tumor markers with

other tumor markers; can obtain information about the growth of tumor [3]. Among cytokeratins, Cytokeratin 19 (CK19) is the smallest cytokeratin because it lacks the C-terminal domain [4]. Molecular weight of this protein is 40 kDa [5]. CK19 is expressed widely in thyroid, breast, colon, and prostate cancer cells [6, 7] and is abundantly found in lung carcinoma [8]. Since this protein is expressed in many tumors, immunohistochemically of CK19 staining is used to diagnose pathological conditions [9]. The aim of this study was to develop the production of a monoclonal antibody against CK 19. This monoclonal antibody has successfully detected CK 19 in lung cancer tissues and a number of cancer cell lines.

Although several commercial CK19 antibodies exist, they often differ in epitope specificity, cross-reactivity profiles, availability, and cost. Developing a monoclonal antibody against a well-defined recombinant CK19 antigen provides an important advantage by enabling the selection of a highly specific hybridoma clone with controlled and validated binding characteristics. Additionally, producing a reliable local CK19 antibody increases accessibility for regional laboratories and reduces dependency on commercial sources, which may be expensive or difficult to obtain. This study aimed to produce and characterize a monoclonal antibody against CK 19 and to evaluate its reactivity in lung cancer tissues and several CK19-expressing cell lines.

Materials and methods

Immunizations

A mixture of 250 µl complete Freund's adjuvant (CFA) (Sigma), 460 µl PBS, and 40 µg CK 19 Recombinant Protein (purchased from American Research Product (ARP) Company) were injected intraperitoneally (IP) to two 6-weeks-old BALB/c mice (purchased from School of Public Health of Tehran University of Medical Sciences, Iran). Two booster injections were administrated every 2 weeks, each containing half amount of recombinant protein used in first injection. 4 days after the last immunization, blood samples were collected and serum were tested by indirect ELISA assays to evaluate polyclonal Ab titers. Both mice were immunized. The last injection was administrated intravenously with 5 µg of recombinant protein was injected.

Hybridoma production

4 days before the fusion, SP2/0 cells were cultured to reach logarithmic growth. The spleen cells from the immunized mouse were fused with SP2/0 cells at a 3:1 ratio by using a polyethylene glycol 1500 solution (Roche Co). The fused cells were suspended in 50 ml HAT medium (Sigma) and then were spread in the plates containing a feeder layer. About a week later, HAT was replaced with HT medium in the wells.

Screening of antibodies

The culture supernatant of clone-containing wells were assessed by indirect ELISA. The Supernatant of 5 clones showed reactivity with the recombinant antigen. After several times rescreening of these clones, two clones were positive. Subsequent limiting dilution of the positive colonies showed that one clone was antibody- secretive.

Isotyping & purification

Type of antibody, heavy and light chains were determined by using an xxx kit (Roche) according to the manufacturer's instructions. For purification of antibodies, clones were first cultured in 25 cm flasks and then transferred to 75 cm flasks. Supernatants were combined with saturated ammonium sulfate with ratio of 1:1 and then centrifuged. The precipitate was dissolved at least in PBS and dialyzed three times. Then antibodies were purified by protein G affinity chromatography according to the manufacturer's instructions.

Affinity measurement

Affinity of purified antibodies were determined using the method of Beatty et al. [10]. Different concentrations (0.5, 0.25, 0.0125, 0.0625 µg/ml) of recombinant CK19 were prepared coated in ELISA wells. After blocking the wells, various concentrations ($\frac{1}{2000}$, $\frac{1}{4000}$, $\frac{1}{8000}$, $\frac{1}{16000}$) of clone 72 purified antibody were prepared in the EIA buffer and added to the wells. After completing the indirect ELISA, absorbance was read at 450 nm. The highest obtained OD was considered as 100% optical density. Others were calculated based on a percentage of the highest OD.

Cross reaction

Specificity of the produced antibody was checked by testing the cross-reactivity with several peptides and recombinant proteins. Compounds that were used to assess cross-reactivity include: NMP22: Nuclear matrix proteins - tumor marker for bladder cancer. CD147: Extracellular Matrix Metalloproteinase Inducer - tumor marker in many cancers. P2-BSA: CD147 peptide. P5-BSA: MUC1 peptide (a large glycoprotein in membrane width and a member Mucin family). P6-BSA: CK19 peptide. BSA: bovine serum albumin. The cross-reactivity of antibodies was assessed by indirect ELISA assay. Volumes of 100 µl from the following concentrations was prepared in ELISA buffer, coated on an ELISA plate and were incubated over night at 37°C. Concentration 1 µg/ml: recombinant protein NMP22, CD147, CK19. Concentration 10 µg/ml: Peptides P2, P5, P6 and BSA. 100 µl from supernatant of clone 72 was added and incubated for 1 h at 37 C∘. After completing the indirect ELISA, absorbance was read at 450 nm.

Table 1 Polyclonal antibody titration of mice

Serum Mice	$\frac{1}{500}$	$\frac{1}{1000}$	$\frac{1}{2000}$	$\frac{1}{500}$
1	> 3.50	3.34	3.21	1.01
2	> 3.50	> 3.50	> 3.50	1.14

Table 2 The first screening

Number of clones	Optical Density
25	≥ 3.00
37	2.5 to < 3
73	2 to < 2.5

Table 3 The absorption rate 5 clones reacted with the CK 19 recombinant protein

The Clones	32	36	41	42	72
Optical density	3.03	2.48	1.99	2.00	3.26

Western blot

The reactivity of produced antibody with lysate of lung tumor tissue and cell lines (MCF-7, HepG2 MDA-MB-468, A431, and HN5) was analyzed by western blot. The lysate of lung tumor tissue and lysate of cell lines were loaded (20 µL/well) on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) and the protein bands were then transferred onto a PVDF membrane (Bio-Rad, USA). The membrane was blocked with skim milk in Tris-buffered saline (TBS) for 120 min at 37 °C, then incubated with monoclonal antibody for overnight at 4 °C, washed three times with 0.1% Tween 20 in TBS, followed by 1.5 h incubation with goat anti-mouse IgG horseradish peroxidase (HRP) at 37 °C. The membrane was washed, then was immersed in diaminobenzidine (DAB) for visualization of bands.

Biotinylation of antibody

Labeling antibodies was performed by linkage covalent to a biotinyl group usually that e typically does not affect antibody binding [11]. Briefly, 0.5 mg of pure antibody was dialyzed against sodium bicarbonate (pH 8.5). The NHS-biotin (Pro Chem, USA) (15µL) in 150µL DMSO (MERCK, Germany) was added to antibody, followed by 1 h incubated in room temperature. Subsequently, 70 µl of 1 M Tris HCl (pH 7.5) was added to the solution, followed by incubation at 4˚C for 1 h. Free biotin was removed by dialyzing three times against 10 mM PBS [12].

Results

Determination of serum antibody titers in mice

The results of polyclonal antibody titration for mice injected with CK19 recombinant proteins summarized in Table 1. Both mice were well immunized against the antigen and significant difference was observed between the serum of immunized and normal mouse.

The results of fusion

Almost all wells in the plates contained clones. Clone screening data was listed in Table 2. Subsequent screening was performed for those clones with high absorption of 2.5. Only five clones reacted with the CK19 recombinant protein. Table 3 shows the absorption rate of the 5 clones. The first cloning by limiting dilution was done for these clones. Table 4 shows results of the first cloning by limiting dilution. Only two clones; derived from the limiting dilution of the numbers 36 and 72; had a positive response to the CK19 recombinant protein. Other clones showed a low absorption rate and thus lost their secretion. Clones 36 and 72 were selected for the second time cloning by limiting dilution. Only sub-clones obtained from Clone Number 72 showed high absorption. Table 5 shows the results of the second limiting dilution. Thus, Clones Number 72 was selected and its properties were determined.

Table 4 The first cloning by limiting dilution

Clones	OD									
32	0.302	0.246	0.360	0.217	0.269	0.229	0.197			
36	0.307	0.223	0.362	0.124	0.276	3.26	0.204	0.325	1.05	0.300
41	0.224	0.286	0.255	0.403	0.314	0.312				
42	0.217	0.245	0.221	0.326	0.271	0.336				
72	0.688	3.5<								

Table 5 The second limiting dilution

clone	OD						
36	0.389	0.123	0.267	0.345	0.165	0.356	165/0
72	3.5<	3.5<	3.5<	3.5<	3.5<	3.5<	3.5<

Fig. 1 Class and subclass and light chain type

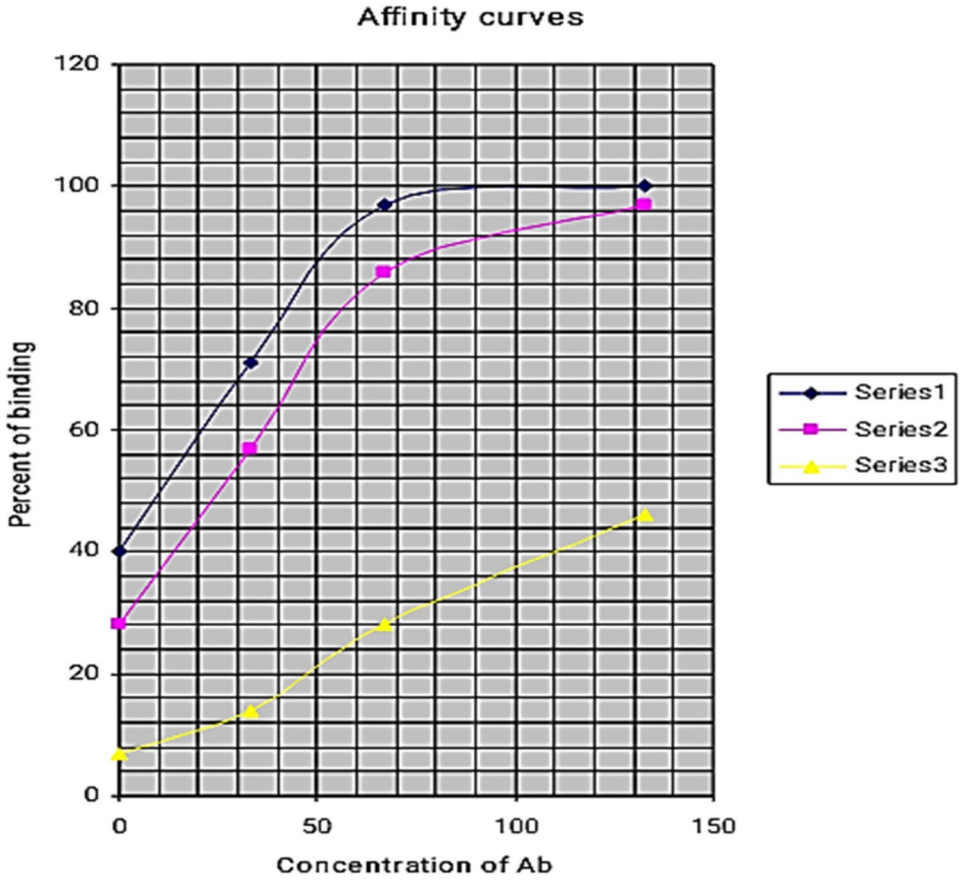

Fig. 2 Percentage of antigen-antibody binding at different concentrations

The results of the class and subclass and type of light chain monoclonal antibody

Class and subclass were determined by using xxx kit. As seen in Fig. 1, this monoclonal antibody was IgG1 with kappa light chain. Figure 2 illustrates the Percentage of antigen-antibody binding at different concentrations.

The results of characteristics determination of the monoclonal antibody (cross reaction)

Table 6 shows results of cross-reacting antibodies with recombinant proteins and peptides.

Determination of antibody affinity

The concentration of the purified antibody was determined by the Bradford method, which was equal to

Table 6 Absorbance obtained from coated various proteins with supernatant of clone number 72

Various proteins	CK19	NMP22	CD147	P2	P5	P6	BSA
OD (Supernatant of clone 72)	OD						
	> 3.50	0.26	0.40	0.22	0.21	0.22	0.20

Table 7 Different concentrations of antibody

Dilutions of purified antibody	$\frac{1}{1000}$	$\frac{1}{2000}$	$\frac{1}{4000}$	$\frac{1}{8000}$
Concentration of the dilution(nM)	1.33	0.67	0.33	0.17

200 µg/ml or 1.33 nmol. The concentration of antibody dilutions was obtained. (Table 7) Maximum Absorbance obtained as a percentage of maximal binding (100%) was considered and other percentages were calculated according to it (Tables 8 and 9). Based on the data obtained from the above table, Fig. 3 – 2 was plotted and the affinity of clone 72 was calculated which was 1.4×10^7 M^{-1}.

$$23\ ?\mathrm{g/ml} = (\mathrm{Ab}')\ ?\mathrm{g/ml}\ 10 = (\mathrm{Ab})\ \frac{[Ag]}{[Ag]} = \frac{1}{0.5} = 2 \quad (1)$$

$$\text{Thus } \mathrm{K_a} = 1.4 \times 10^7\ \mathrm{M}^{-1} \quad (2)$$

Determination of the molecular weight of antigen by Western blotting

The results of Western blot analysis for lysate of lung tumor tissue and lysate of cell lines (MCF-7, HepG2 MDA MB 468, A431, HN5) are shown in Figs. 3 and 4. Antigen had a molecular weight of about 40 kDa. To assess the effects of antibody biotinylation on western blotting quality, purified antibody was biotinylated and the results of the western blot is presented in Fig. 5.

Discussion and conclusion

Cytokeratin 8, 18 and, 19 are the most abundant cytokeratins in simple epithelial cells [13]. Cytokeratins can be present in the circulation either as single pieces or as small and large protein complexes. Levels of these proteins in the blood are low in healthy individuals.

Whereas elevated concentrations are often found in the patients with carcinomas [14]. The study of El Bastawisy et al. in 2011 studied patients with benign and malignant lung tumors. It was found that CK19 is a reliable marker for diagnosis and prognosis of lung cancer in advanced stages (14In 1995, Wieskopf et al. examined a number of tumor markers in patients with lung cancer and lung disease and revealed that the sensitivity and the specificity of soluble CK19 fragment for non-small cell lung cancer is higher than other tumor markers such as CEA, SCC and NES [15]. In the present study, reactivity of antibody with lung tumor tissue and cell lines MCF7, MDA-MB-468, HepG2 were assessed. ommercial suppliers such as Biorbyt and Proteintech report similar additional bands between 45 and 55 kDa in CK19-positive cell lines (e.g., MCF-7, HepG2, HT-29), supporting the likelihood that the band observed in our study represents a modified or alternative CK19 form. The aim of this study was to assess the production of a monoclonal antibody against CK19. The MCF7 and HepG2 cell lines are used as a positive control in Western blot by

Table 8 Absorbance of different concentrations of coated CK 19 recombinant protein, in the presence of various concentrations of antibodies

The concentration of the purified antibody clone 72(nM)	coated concentrations of CK 19 Recombinant Protein (µg/ml)		
	0.5	0.25	0.125
	OD		
1.33	1.75	1.70	0.8
0.67	1.70	1.5	0.5
0.33	1.25	1	0.25
0.17	0.7	0.5	0.12

Table 9 Binding percentages of various concentrations of antigens and antibodies

The concentration of the purified antibody clone 72(nM)	coated concentrations of CK 19 Recombinant Protein (µg/ml)		
	0.50	0.25	0.125
	OD		
1.33	100	97	46
0.67	97	86	28
0.33	71	57	14
0.17	40	28	7

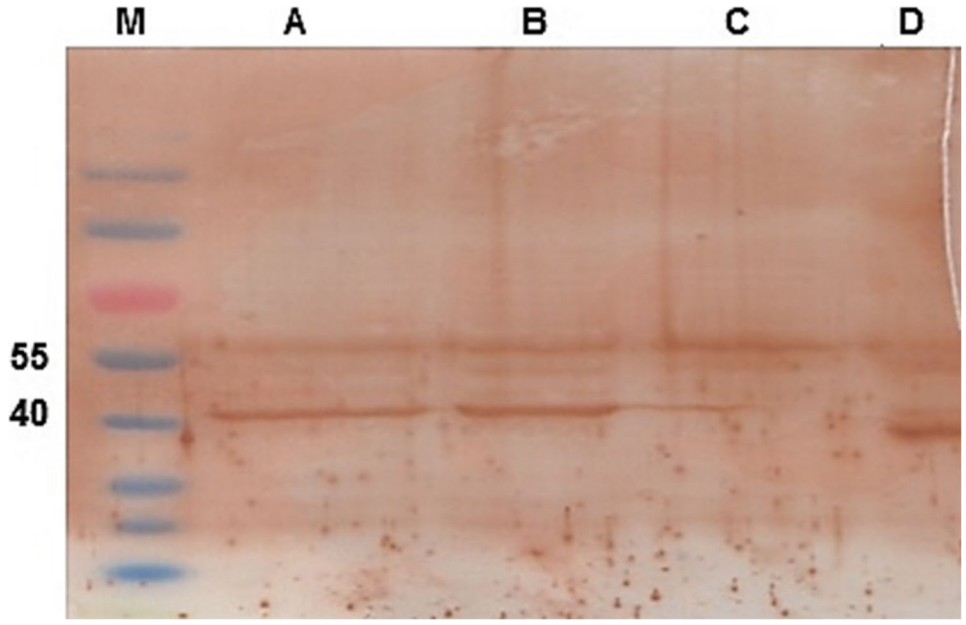

Fig. 3 The results of western blot analysis. M: molecular weight marker, A: MCF-7, B: MDA-MB-468, C: HN5, D: lung tumor tissue

most companies. The results of Western blotting with our antibody demonstrated the presence of two bands at molecular weights of 40 and 55 kDa. To confirm the specificity of the bands, Purified antibody was biotined and used in western blot analysis. In this case, avidin-mediated HRP binds to antibody and the reaction is more specific and cross reactivity of the secondary antibody with the sample components did not occur. The results of this method also revealed the bands of 40 and 55 kDa.

In the present study, we did not perform a direct, side-by-side comparison of our monoclonal antibody with commercially available CK19 antibodies by Western blot. However, according to the Western blot data provided by several commercial suppliers (e.g., Abcam, Biorbyt, Proteintech), additional bands in the 45–55 kDa range are observed in cell lines such as MCF-7, HepG2, and HT-29 when probed with anti-CK19 monoclonal antibodies. These data are in line with our observation of a ~ 55 kDa band and support the possibility that this band may correspond to other cytokeratin family members and/or post-translationally modified forms of CK19. Nonetheless, the lack of an experimental head-to-head comparison with commercial antibodies represents a limitation of our study, and future work will include such comparative analyses to further validate the specificity of the 55 kDa band.

Since the range of molecular weight of cytokeratins are 40 kDa to 67 kDa [16, 17] and because CK 19 is the smallest cytokeratin with molecular weight of 40 kDa, therefore the 40-kDa band observed in Western blot analysis is related to CK 19 .Among cytokeratins, one type, the α-helical domains shared 50–90% sequence identity, while the cytokeratins of two different types displayed approximately 30% homology in these regions [18]. Bands observed at 55 kDa could be due to cross-reactivity of the antibody with other cytokeratins. Another hypothesis for the existence of bands other than of 40 kDa is post-translational modifications of cytokeratins such as phosphorylation and glycosylation. These modifications are made in the N and C terminal of cytokeratins [19–22] that could lead to an increase in molecular weight. Other bands were observed in the Western blot of some commercial monoclonal antibodies in addition to the 40 kDa band [21]. The higher molecular weight band (~ 55 kDa) observed in Western blotting is likely due to post-translational modifications or interaction with other cytokeratins, as similarly reported by commercial antibodies. For example, EPR1579Y antibody; made by company Abcam; a strong band was observed in the region of 50 – 37 kDa. A monoclonal antibody against CK 19 has been produced by the biorbyt company. Western blotting with HT-29 cell line demonstrated a band of upper than 43 kDa. The protein tech company for its monoclonal antibody has done western blot with HepG2 and MCF7 cell lines. Result of HepG2 demonstrated a band of lower than 45 kDa and MCF7 bands of 45 kDa and higher were observed for MCF7. A431 cell line is epidermoid carcinoma, since CK 19 does not exist in the epidermis, therefore no band was observed in A431 cell line.

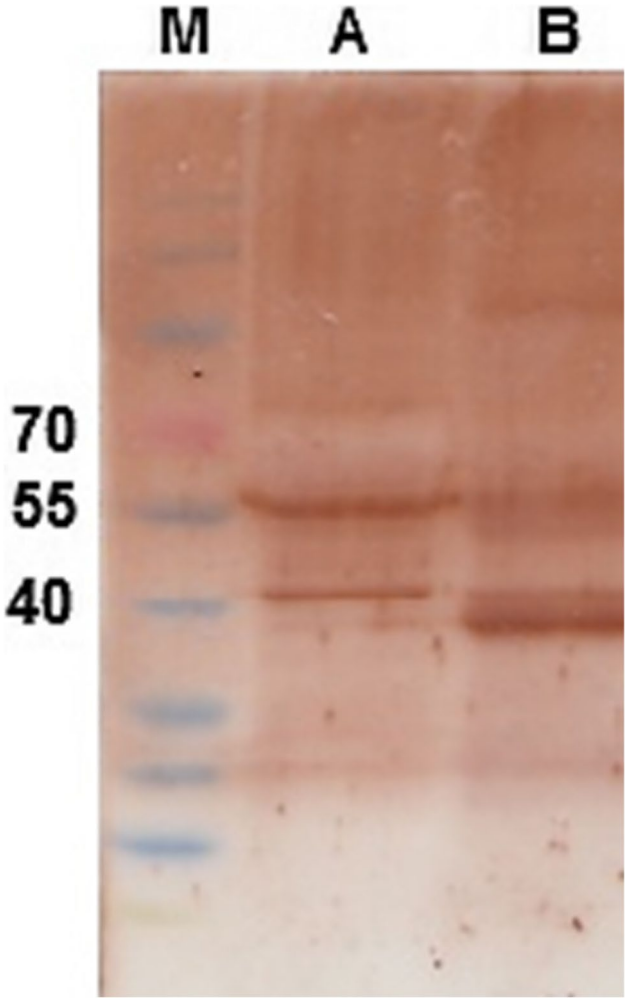

Fig. 4 The results of western blot analysis. M: molecular weight marker, A: HpeG2, B: lung tumor tissues

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12865-025-00792-7.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

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Authors' contributions
M. A.M.M.: wrote the main manuscript.J. M.: investigated the obtained results; H. G.: Formal analysis; S. K.:SoftwareN. M.: prepared all figuresA. D.: supervisorG. Ö.: edited the final draft; Y.D.: edited the final draft; S.T.H. Investigated the revised draft and edited the final version.All authors reviewed the manuscript.

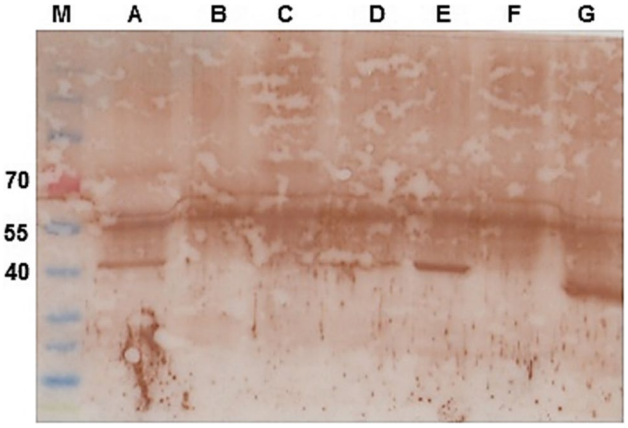

Fig. 5 The results of the Western blot with biotin antibodies. M: molecular weight marker, A: HpeG2, B: Hc1, C: A431, D: MCF-7, E: MDA-MB-468, F: HN5, G: Lung tumor Tissue. Western blot analysis of CK19 expression in lung tumor tissue and cancer cell lines. Lysates (20 µL) were resolved by SDS-PAGE and transferred to PVDF membranes. The membrane was incubated with the monoclonal antibody overnight (4 °C, 1:1000 dilution) and detected using HRP-conjugated goat anti-mouse IgG. Enhanced contrast and reduced background were applied for clarity

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Data availability
The datasets generated and supporting the findings of this article are obtainable from the corresponding author upon reasonable request.

Declarations

Ethic approval and consent to participate
The Committee of Medical Biochemistry, Faculty of Medicine, Cukurova University, Adana, Turkey, approved the experimental procedures used in this study (approval no. TUR-K450-123) on Month 08, 2025.

Consent for publication
Not applicable

Competing interests
The authors declare no competing interests.

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