



# Monoclonal Antibody Production Against Vimentin by Whole Cell Immunization in a Mouse Model

Marzieh Rezaei<sup>1</sup> and Abbas Ghaderi<sup>2\*</sup>

<sup>1</sup> Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

<sup>2</sup> Department of Immunology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

\*Corresponding author: Abbas Ghaderi, Shiraz Institute for Cancer Research, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran, Tel: +98- 7132303687, Fax: +98-7132304952, E-mail: ghaderia@sums.ac.ir

Received: 15 Jan. 2017; Revised: 30 Dec. 2017; Accepted: 10 Jan. 2018; Published online: 15 May 2018

**Background:** Pancreatic carcinoma is the fourth-leading cause of cancer death in the United States and due to its late presentation, only few patients would be candidates for the curative treatment of pancreatotomy. Monoclonal antibodies have brought hope to targeted therapy.

**Objectives:** To identify new biomarkers, a panel of monoclonal antibodies was generated against newly established cell line, Faraz-ICR from a patient with pancreatic acinar cell carcinoma.

**Material and Methods:** Balb/c female mice were immunized with Faraz-ICR cell line and their splenocytes fused with SP2/0 myeloma cell line. Highly reactive hybridoma producing antibodies against Faraz-ICR was detected using ELISA, immunofluorescence staining and flow cytometry. Western blot and 2D immunoblot were utilized for further characterization of the target antibodies.

**Results:** Among highly reactive clones, the reactivity of 7C11 clone was assessed in comparison to other epithelial tumors. The antibody isotype was IgM that reacted with a 55 kDa protein in western blot analysis. To further characterize the target antigen, immunoproteome of the Faraz-ICR cell line was performed. By LC-MS analysis, the target of 7C11 clone was identified to be vimentin.

**Conclusions:** Pancreatic cancer is a highly lethal malignancy with no reliable biomarker for early detection and diagnosis. In this study, by establishing a pancreatic acinar carcinoma cell line, a panel of monoclonal antibodies was generated to identify specific or associated cancer targets. Furthermore, 7C11 mAb was introduced that can specifically recognizes vimentin as a tumor marker. This antibody may serve as a new tool for prognostic and therapeutic strategies.

**Keywords:** Hybridoma, Monoclonal antibody, Pancreatic acinar cell carcinoma, Vimentin

## 1. Background

Monoclonal antibodies (mAbs) have extensive and precise applications in medical research, diagnostics and treatment of diseases (1, 2). In cancer biology, mAbs are of particular interest, providing valuable information in different areas such as antigen characterization and localization, protein interactions, vaccine production, and direct targeted therapy strategies (3-6). In different malignant tumor types such as pancreatic and colon cancers, mAbs have led to promising results; targeting certain proteins that are involved in tumor growth, proliferation and metastasis (7, 8). Accordingly, use of

mAbs can be considered as a therapeutic strategy.

Vimentin, widely expressed mammalian intermediate filamentous (IF) protein, is expressed by normal mesenchymal cells. It is considered as the marker for differentiation of mesenchymal cells due to its constitutive expression (9). Vimentin is a hallmark of epithelial to mesenchymal transition (EMT), a cellular reprogramming process in which epithelial cells loss their polarity, exhibit increased motility and acquire a mesenchymal phenotype. These ~ 57 kDa protein is structurally highly conserved and belongs to type III IF family (10). The corresponding gene is being expressed

in numerous other cells such as fibroblasts, endothelial linings of blood vessels, renal tubular cells, macrophages, neutrophils, and leukocytes (9). Vimentin is an structural protein that maintains cell and tissue integrity through generating a cellular scaffold (11). Under stress conditions, vimentin functions as a signaling protein that is involved in survival, adhesion and migration in addition to its structural properties, (12, 13).

Here, vimentin specific mAbs was developed for tracking purposes and development of new biomarkers of tumor cells.

## 2. Objectives

Specific mAb clones were generated by whole cell immunization with high affinity for pancreatic cancer cell line. One of which was introduced here, have a high affinity for vimentin.

## 3. Materials and Methods

### 3.1. Cell line Establishment and Culture

Faraz-ICR cell line was derived from a tumor specimen surgically gained from a 58-year old female patient with primary pancreatic acinar cell carcinoma by the collagenase digestion protocol in the Institute for Cancer Research, Shiraz, Iran (16). After establishment and stability of Faraz-ICR cell line in the medium, it was used for mouse immunization as the antigen pool. Faraz-ICR cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, USA) and 1% (w/v) penicillin/streptomycin at 37 °C in a humidified atmosphere with 5% CO<sub>2</sub>. Cells at passages 75 until 100 were used for mouse immunization.

### 3.2. Cell Preparation and Mouse Immunization

#### Procedure

Balb/c mice (female, 6-8 week old) were immunized with five consequent intraperitoneal injections. Each injection contained 10<sup>7</sup> cells detached with scraper and washed twice with cold phosphate buffer saline (PBS) with a two-week interval between each immunization. Mouse serum titration was performed with Enzyme-linked Immunosorbent Assay (ELISA). Upon proper titration, three boosters were carried out (11 weeks after initiation of immunization).

### 3.3. Generation and Selection of Hybridoma

Three days after the last injection, spleen cells from the immunized mice were fused with mouse myeloma SP2/0 cells (National Cell Bank of Iran, Pasteur Institute of Iran, Tehran). For this stage, mouse myeloma Sp2/0

cells were cultured and proliferated in RPMI-1640 medium (Gibco, USA) plus 10% FBS. Spleenocytes and myeloma cells were mixed at a ratio of 5:1, and the mixture was washed twice with pre-warmed RPMI-1640. Fusion was performed using pre-warmed polyethylene glycol (PEG) 1500 (Sigma-Aldrich, Germany). The resulting hybridoma cells were dispensed at 2 × 10<sup>5</sup> cells per well in 96-well plates and cultured in RPMI medium containing 20% FBS, 1% penicillin/streptomycin, 1% sodium pyrophosphate and 1% NEAA (both from Gibco, USA) and combined with HAT supplement (Sigma, USA). Following fusion (14 days), the reactivity of culture supernatants from wells containing hybrids was identified by ELISA. Positive hybridomas were cloned by limiting dilution process using the conventional methods and sub-cloned and rescreened by ELISA and immunocytochemistry.

### 3.4. ELISA Screening

Mouse serum titrations and screening of hybridoma supernatants were performed by ELISA. Faraz-ICR cells were washed twice with cold PBS and lysed in the urea buffer (7 M urea, 2 M thiourea, 4% Chaps, 80 mM DTT, 1% IPG buffer) and protein concentration was determined with Bradford protein assay (Bradford, 1976). The wells of the ELISA plate (Nunc, Denmark) were coated with 5 µg/well of cell lysate dissolved in the bicarbonate buffer (pH 9.4) and incubated overnight at 4 °C. To avoid non-specific binding, the plates were blocked with 1% BSA in PBS-T. Hybridoma supernatants were added for 1.5 h at 22 °C and washed three times with PBS-T. The plates were washed three times with PBS-T and were incubated for 1 h at 22 °C with secondary HRP-conjugated anti-mouse IgG antibody (Sigma, USA) at a dilution of 1:2000. After 5 washes with PBS-T, immunoreactivity of the reaction revealed with (DAB, Gibco, USA) substrate and finally, the OD was determined by an ELISA reader at 450 nm.

### 3.5. Mass Production of Monoclonal Antibodies in Ascites Fluid

For mass production of antibody, first 0.5 mL pristane (Sigma, USA) was injected intraperitoneally into Balb/c mice (female, 6-week old). One week after priming, 5 × 10<sup>6</sup> cells of the 7C11 clone in 0.5 mL PBS were injected intraperitoneally into each mouse. Mice were sacrificed after 7-10 days, and ascites fluid was sucked from their peritoneum using a needle.

### 3.6. Immunofluorescence Staining

Different cell lines containing pancreatic cell lines, breast cell lines and mesenchymal stem cells were grown at 3 ×

$10^4$  on four-well chamber slides for 24 h. After the wash with PBS, the cells were fixed with cold methanol for 10 min, permeabilized, and blocked with 1% BSA in PBST containing 1% Triton X-100 for 45 min at 22 °C. Slides were incubated with 7C11 clone culture supernatant as a primary monoclonal antibody for 16 h at 4 °C. Slides were washed 3 times with PBST and further incubated with secondary fluorescent FITC-conjugated antibody: Goat anti-mouse (Santa Cruz, USA) in 1.5% BSA in PBST for 2 h at 22 °C. After extensive wash, 7C11 mAb reactivity and target localization of antigens were visualized using a fluorescent microscope ni-80 (Nikon, Japan).

### 3.7. Western Blot Analysis

For determination of 7C11 mAb specificity and appropriate concentration for 2D immunoblotting, Western blot was performed. Lysates of the cell lines (Faraz-ICR, Patu-8902 and mesenchymal stem cells) were prepared in the 8 M urea buffer. Cell lysate (30 µg) was run on a 12% SDS-PAGE and electrophoretically transferred to PVDF membranes by semi-dry blotter (Trans-Blot<sup>®</sup> SD Semi-Dry Electrophoretic Transfer Cell, Bio-Rad, Hercules, CA, USA). The membranes were blocked for 16 h at 4 °C with a blocking solution containing 5% skimmed milk (Fluka, Sigma, USA) in TBST (Tris buffer containing 1% Tween-20), and were incubated with mouse ascites at a different concentration as primary antibody (overnight at 4 °C). After washing with TBST, the membrane was incubated for 1 h at 22 °C with HRP-conjugated anti-mouse antibody (Sigma, USA). Following the wash with TBST, the membrane was developed by treatment with ECL and X-ray film as routine method.

### 3.8. 2D-Immunoblotting

Faraz-ICR cell pellets were resuspended and lysed in lysis buffer (urea and thiourea buffer) as described above. For the first dimension, 400 µg protein lysate was loaded onto IPG Dry strips (pH 3-10 NL; 18 cm, GE Healthcare, USA) in gel rehydration for 18 h at 50 V. Isoelectric focusing (IEF) was performed on an IPGphor IEF unit system (Bio-Rad, Hercules, CA, USA) for a total of 55000 Vh. Prior to SDS-PAGE, the IPG strips were equilibrated in two steps (first, 15 min with a solution of Tris/HCl buffer (5 mM; pH 8.8), urea (6 M), 30% glycerol, 2% SDS, and 2% DTT, and followed by a further 15 min in the same buffer containing 2.5% iodoacetamide). For the second dimension, strips were run on 12% SDS polyacrylamide gel at a 30 mA constant current. Protein spots were visualized by a modified Coomassie Brilliant Blue staining method. For 2D-immunoblotting, the 2D gel was transferred across the PVDF membrane by a semi-

dry blotter (Bio-Rad, Hercules, CA, USA) for 1.5 h and followed by routine Western blot procedure for detecting the 7C11 clone target in the Faraz-ICR cell lysate. In this process, 7C11 clone ascites fluid was used as a primary antibody. Immunoreactive protein spots were manually cut from 2D gels stained with Commassie and sent for MALDI-TOF/TOF MS analysis to the United Kingdom (Department of Biology, The Proteomics & Analytical Biochemistry Labs, and University of York, UK).

### 3.9. Isotype Determination

The isotype of 7C11 mAb was determined according to the Mouse Ig isotyping ELISA kit manual (eBioscience, Vienna, Austria). Briefly, capturing antibodies (rat anti mouse IgG1, IgG2a, IgG2b, IgG3, IgA, IgM, Kappa and lambda light chain) at 1:250 dilution in the coating buffer were coated in the wells and incubated for 16 h at 4 °C. After blocking, culture supernatant and ascites fluid of the 7C11 clone were added to each well for 2 h. The plate was washed and HRP-conjugated anti-mouse Ig was added as detection antibody (1:250 dilution, 1 h). Finally, the reactivity of the wells was measured by adding substrate solution and OD was read at 450 nm (Test repeated 3 times).

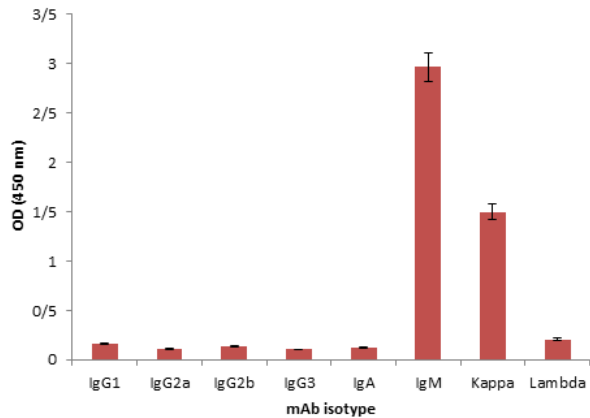
### 3.10. Flow Cytometry

The reactivity of 7C11 mAb with different cells was verified by flow cytometry. After routine culture in flasks, cells were detached and dissociated to single cells with trypsinization and washed twice with cold PBS. For intracellular staining, the cells were fixed with paraformaldehyde solution 2% and permeabilized by perm/wash solution (BD, USA). The cells were distributed per tubes and stained with 7C11 clone ascites fluid as the primary antibody at 1:10000 dilution for 30 min at 4 °C. Following 3 consecutive washing with PBS, FITC conjugated sheep anti-mouse Ig (Avicenna Research Institute, Tehran, Iran) (1:50) was added to the cells and incubated for 45 min in dark at 4 °C. Finally, three washes with PBS were performed and marker expressions were assessed with FACScalibur flow cytometer. Surface staining of the cells was carried out as intracellular staining without fixation/permeabilization steps. The expression of the target protein was analyzed using FlowJo software version 7.6.2 (Tritar Inc., USA).

## 4. Results

### 4.1. Production and Characterization of a Novel Anti-vimentin mAb

To obtain new antibodies for tumor markers, Balb/c mice were immunized with Faraz-ICR cell line.



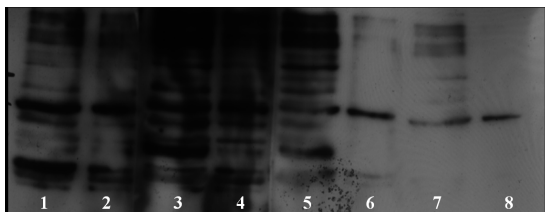
**Figure 1.** Isotype determination of 7C11 mAb by ELISA.

Splenocytes from the mouse with the highest titration were fused with myeloma SP2/0 cells for stable production of mAb. 7C11 was amongst the clones with high reactivity as proved by ELISA of Faraz-ICR lysate. Following cloning and sub-cloning, (four times), selected clones in each step tested with ELISA. The culture supernatant from the selected hybridomas were further characterized.

The isotype determination of 7C11 mAb revealed that the heavy chain was IgM, and the light chain was kappa (**Fig. 1**). The results of ELISA were confirmed by immunocytochemistry. After cloning and sub-cloning, the clone with highest and most stable antibody production was selected. The clone was used to produce antibody in culture media and ascites fluid.

#### 4.2. Western Blot Analysis

Western blot analysis demonstrated that 7C11 mAb recognizes a target of ~55 kDa protein band in Faraz-ICR and Patu-8902 cells (**Fig. 2**). In addition, western blotting with different concentrations of 7C11 mAb as primary antibody determined the appropriate

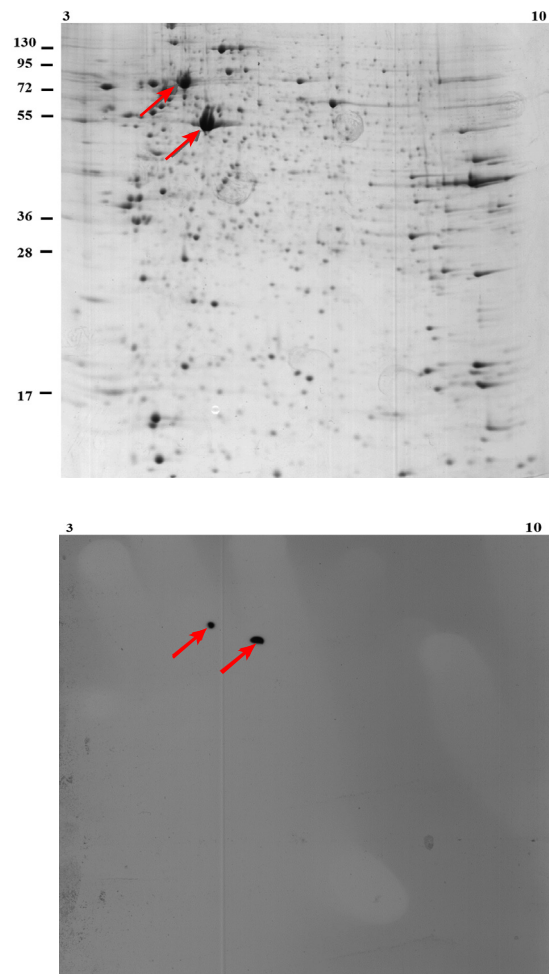


**Figure 2.** Western blot analysis of 7C11mAb reactivity with pancreatic cancer cell lines' lysates, lane1: Faraz-ICR and lane 2: Patu-8902, mAb concentration 1:500, lanes 3 and 4, repeated with mAb concentration 1:1000, lanes 5 and 6, repeated with mAb concentration 1:2500, lane 7 and 8, repeated with mAb concentration 1:5000.

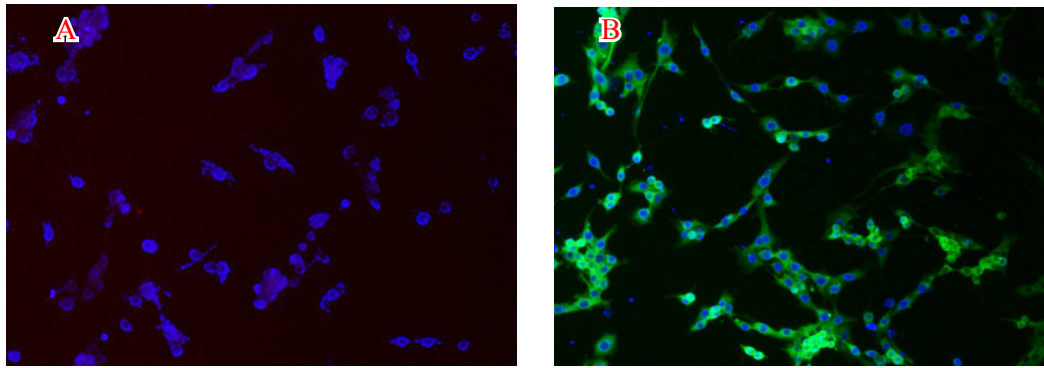
concentration of mAb for 2D immunoblotting and its target.

#### 4.3. 2D-Immunoproteome

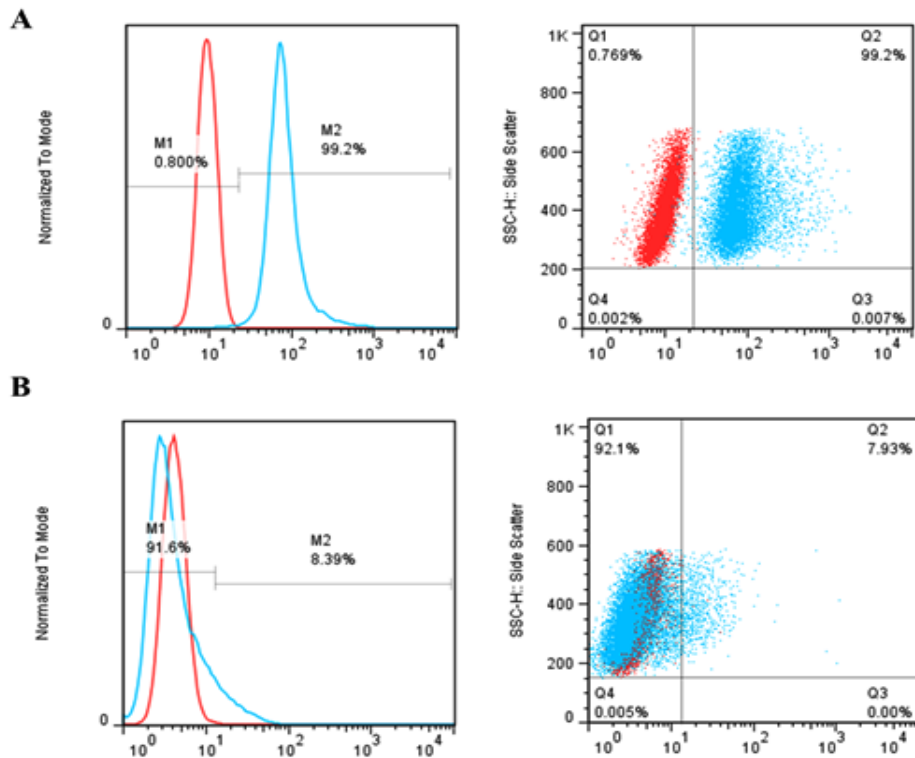
The result of western blot analysis showed that 1:5000 dilution of 7C11 mAb ascites fluid is suitable for obtaining a unique band in immunoblotting. The same concentration was used to determine the target spot in 2D-immunoproteome. The results of tandem mass spectrometry were searched in the National Center for Biotechnology Information (NCBI, USA) database, using the Mascot search engine (Matrix Science, UK). MS analysis revealed a significance score of 496 that 7C11 clone recognized vimentin (nominal mass: 53676 Da and calculated  $pI$ : 5:06). These outputs are consistent with the results obtained from 2D-immunoblot and western blot analysis (**Fig. 3**).



**Figure 3.** A) 2D proteome of Faraz-ICR cell line stained with Coomassie Brilliant Blue, B) 2D immunoproteome of Faraz-ICR cell line stained with 7C11 mAb as a primary antibody. Arrows show vimentin spots in proteome and immunoproteome.



**Figure 4.** Immunofluorescent staining of Faraz-ICR with 7C11 mAb and FITC-conjugated anti mouse as secondary antibody ( $\times 100$ ). A) Negative control, without staining with primary antibody, B) Faraz-ICR cell stained with 7C11 mAb.



**Figure 5.** Immunostaining of Farz-ICR cell line by flow cytometry, histogram and dot blot charts, A) intra-cellular staining, B) surface staining, red: isotype staining, blue: 7C11 mAb staining.

**4.4. Immunofluorescence Staining and Flow Cytometry**  
 This antibody was characterized by immunofluorescence staining of Faraz-ICR cells. After fixation and permeabilization, cells were blocked and probed with 7C11 mAb followed by FITC-conjugated secondary antibody. It became clear that the 7C11 mAb identified the target molecule well, when compared to the negative control (Fig. 4).

To verify whether the 7C11 clone detected vimentin in normal structure, flow cytometry with normal and cancerous cell lines such as Patu-8902, Mia-paca2, MCF-7, Pari-ICR, SW48, PBMC, Jurkat and Raji was performed. Flow cytometric analysis showed that 7C11 mAb recognizes intracellular vimentin molecules in different cells (Fig. 5). Hence it can recognize its target in natural form with high affinity.

## 5. Discussion

Nowadays, antibody-based therapy for cancer is one of the most prosperous and promising therapeutic strategies in different tumor types and in some era has led to successful clinical trials with notable and acceptable results (17). In this regard, the definition of cell surface antigens in tumors, including molecules that are overexpressed, mutated or selectively expressed, are considered to be interesting. Due to high selectivity and specificity, mAbs have a significant role in targeted therapies with some advantages over genomics and proteomics approaches (18,19).

Whole cell immunization leads to production of mAbs with different specificity and diversity for proteins with native conformations and modifications (20). Consequently, we can select specific mAbs from this panel. The use of new cell lines as an antigen pool for mAb generation could be more useful due to fewer changes in comparison to native forms (21). Using a panel of antibodies and functional assays, we were able to verify that Faraz-ICR cell line has an epithelial nature with some aspects of EMT and aggressive phenotype (16). As a result, it could be a suitable candidate for mouse immunization and mAb generation. Expression or elevated level of vimentin in pancreatic cancer cells is an indisputable fact (22) and Faraz-ICR cell line can be an appropriate source for mAb production for this molecule.

Poor prognosis, early metastasis, late diagnosis and resistance to chemotherapy agents are the main challenges of pancreatic cancer (23). For these reasons, new approaches for finding novel biomarkers are inevitably required (24). To discover novel specific tumor antigens expressed on pancreatic cancer cells, a panel of mAbs was generated against the newly established Faraz-ICR cell line using the mouse immunization with whole cell. Among them, some clones were obtained that recognized their targets with high affinity in normal and transformed cells. Accordingly, some of these target molecules were checked to determine their identity. One of these clones was 7C11 with high affinity for vimentin molecule.

7C11 mAb recognized vimentin in normal and cancerous cells in both non-reducing and reducing forms. As a result, 7C11 mAb can be used for vimentin targeting in different diagnostic and therapeutic processes. These results were confirmed with immunofluorescence staining and flow cytometry. Moreover, overexpression of vimentin was clearly evident in tumor cells compared to normal cells such as peripheral mononuclear and mesenchymal cells, as confirmed in other studies (25, 26).

Vimentin belongs to the intermediate family of proteins, which involves in maintenance of cell shape, integrity and stabilization of cytoskeletal interactions. It was shown that vimentin is associated with nucleus, mitochondria and ER (27, 28). It is a marker for tissues with mesenchyme nature, but irregularly can be expressed by metastatic epithelial neoplasms (29). These features often correlate with poor survival and high metastasis in some tumor types such as pancreatic cancer (30). These carcinoma cells with markers of mesenchymal differentiation have different biological and clinical behavior. Cytoplasmic vimentin is overexpressed during EMT and is associated with metastasis, invasion, and proliferation (31). Moreover, vimentin transportation to the cell surface was seen in some malignancies (10). The reason for this phenomenon is unclear, but it has a positive correlation with disease progression, resistance to chemotherapeutic reagents and formation of stable colonies (32). Vimentin, as a member of adhesion networks, is a hallmark of EMT during tumor progression and could help neoplastic cells to metastasize, and promote tumor progression (33). Due to the important role of vimentin, targeting this polypeptide leads to cell dysfunction (34). Besides, changes in its expression levels or its aberrant expression in other tissues, makes it a good therapeutic target (35). Production of specific antibodies to vimentin for both diagnosis and treatment of cancer is in progress by different laboratories (10, 36). Furthermore, considering the importance of this molecule, production and characterization of new mAbs is worthwhile.

Here, an stable clone (7C11) with capability to produce mAb specific for vimentin was obtained through whole cell immunization method of mouse by injecting pancreatic cancer cell line. This clone, 7C11, was efficiently recognized vimentin in normal and cancerous cells. Thus, it can be used for prognostic, therapeutic, and probably diagnostic purposes.

## Acknowledgements

This work was financially supported by Shiraz Institute for Cancer Research (Grant No. ICR-100-508).

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