The Association of Cell Surface Fibromodulin Expression and Bladder Carcinoma

Ali Ahmad Bayat[1], Niloufar Sadeghi[1], Ali Salimi[1], Ghazaleh Fazli[1], Mohammad Reza Nowroozi[2], Solmaz Ohadian Moghadam[2], Amin Radmanesh[3,4], Mohsen Tabasi[3,5], Ali Reza Sarrafzadeh[6], Omid Zarei[7], Hodjattallah Rabbani[1]*

Purpose: Fibromodulin (FMOD) is a secretory protein which is considered a major component of extracellular matrix. Its dysregulation in different types of cancer implies it as a promising target for cancer therapy. Within the scope of its rather wide expression in different tumors, we studied the expression of FMOD and the effect of anti-FMOD antibody in bladder cancer cells in order to identify new target for diagnostic and therapeutic interventions. We report here for the first time the expression of FMOD in bladder cancer cell lines in comparison to the normal cell line and tissues.

Methods: A peptide-based produced anti-FMOD murine monoclonal antibody (mAb) (clone 2C2-A1) was applied for evaluation of FMOD expression in bladder cancer and normal tissues by immunohistochemistry (IHC) staining. Furthermore, the expression of FMOD was examined in human bladder cell lines, 5637 and EJ138, as well as a non-cancerous human cell line, human fetal foreskin fibroblast (HFFF), by immunocytochemistry (ICC) and flow cytometry. The apoptosis induction of anti-FMOD mAb was also evaluated in bladder cancer cells.

Results: IHC and ICC analyses revealed that the qualitative expression of FMOD in bladder cancer tissues and cell lines is higher than in normal tissues and cell lines. Flow cytometry analyses revealed that 2C2-A1 mAb could recognize FMOD expression in 84.05 ± 1.85%, 46.1 ± .4% , and 2.56 ± 1.26% of 5637, EJ138, and HFFF cells, respectively. An effective apoptosis induction was detected in 5637 and EJ138 cells with no significant effect on HFFF cell.

Conclusion: To our knowledge, this is for the first time reporting surface expression of FMOD in bladder cancer. This significant surface expression of FMOD in bladder cancer with no expression in normal bladder tissues and the capacity of inducing apoptosis through directed targeting of FMOD with specific monoclonal antibody might candidates FMOD as a diagnostic marker as well as a potential immunotargeting with monoclonal antibody.

Keywords: bladder cancer; fibromodulin; flow cytometry; monoclonal antibody

INTRODUCTION

One of the major obstacles in combating bladder cancer is to identify new specific markers for targeted therapy and diagnosis. Although several markers such as UroVysion, NMP22 (Nuclear Matrix Protein 22), BTA (Bladder Tumor Antigen), and ImmunoCyt/uCyt+[(1)] have shown their specificity in diagnosis and treatment of bladder cancer but still their function and effectivity are not enough good.

Regardless of several strategies for bladder cancer therapy[(2)], about 5,490 new cases and 200,000 deaths were reported annually worldwide. This high rate of incidence and mortality highlights the importance of novel therapies for the treatment of bladder cancer[(3)].

Recently, we have introduced two novel targets of ROR1[(4)] and sortilin (under review), highly expressed in bladder tumors. Both markers have a cell surface expression with different functions. ROR1 plays an important role in proliferation, differentiation, metastasis, and polarization[(5)]. In contrast, sortilin is functioning as a sorter in cytoplasm with only less than 10% surface expression[(6)]. Apparently, the functional property and cellular localization have an effect on cellular function in normal and pathological conditions. To add additional markers to the list, we have sought the functional role of fibromodulin in this cancer.

Fibromodulin is a member of small leucine-rich repeat

[1]Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran.
[2]Uro-Oncology Research Center, Tehran University of Medical Sciences, Tehran, Iran.
[3]Legal Medicine Research Center, Legal Medicine Organization, Tehran, Iran.
[4]Department of Tissue Engineering and Applied cell sciences, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
[5]Molecular biology Unit, Pasteur Institute of Iran, Tehran, Iran.
6 Department of Pathology, Khatam Al Anbia Hospital, Tehran, Iran.
7 Cellular and Molecular Research Center, Research Institute for Health Development, Kurdistan University of Medical Sciences, Sanandaj, Iran.
*Correspondence: Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, IRAN
P.O. Box: 19615-1177
Tel: +98 21 22432020.Fax: +98 21 22432021
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Table 1. Flow cytometry on bladder cancer and normal cell lines

Cell line	Antibody	MFI[b]	POP[c]	MFI×POP
EJ138	Anti- FMOD mAb[a]	19.3	46.6	899.38
	Isotype control	11.1	3.34	37.07
5637	Anti- FMOD mA[b]	43.4	85.9	3728.06
	Isotype control	33.7	8.72	293.86
HFFF	Anti- FMOD mA[b]	2.64	1.37	3.61
	Isotype control	1.39	0.862	1.2

[a] Monoclonal antibody
[b] Mean fluorescence intensity
[c] Percentage of positivity

proteoglycans (SLRPs) family and also is an important component of extracellular matrix (ECM)[7]. FMOD gene encodes a 42-80 kDa protein in different types of connective tissues such as cartilage, sclera, tendon, skin and cornea[8], and also a 55-75 kDa protein in chronic lymphocytic leukemia (CLL)[9].

FMOD has significant roles in various physiological processes such as angiogenesis, regulation of transforming growth factor beta (TGF-β) activity, apoptosis, differentiation of human fibroblasts into pluripotent stem cells, and inflammatory mechanisms. Also FMOD has been considered as a new tumor-related antigen[10]. The cell surface expression of FMOD has been studied in several cancers including B-CLL[11,12], prostate cancer[13], and glioblastoma,[14] with a lack of comprehensive study in bladder cancer. The assessment of FMOD surface expression in bladder cancer cells might introduce FMOD as a diagnostic and therapeutic target[9, 11,15,16]. In this study we used ICC, IHC, and flow cytometry techniques to explore the expression profile of FMOD and its functional role in bladder cancer in order to find a novel diagnostic method as well as a novel target to combat this malignancy.

MATERIALS AND METHODS

Cell culture

Human bladder cancer cells lines, EJ138 (Invasive transitional bladder carcinoma) (NCBI Code: C429; ECACC Number: 85061108), 5637 (Non-invasive grade II transitional bladder carcinoma) (NCBI Code: C450; ECACC Number: DSMZ NO: ACC 35) and human normal cell HFFF (Human caucasian Fetal Foreskin Fibroblast) (NCBI Code: C107) cells were purchased from National Cell Bank of Iran (Pasteur Institute, Tehran, Iran). All cell lines were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA), containing 10% fetal bovine serum (FBS) (GIBCO Invitrogen, USA), penicillin (100 U/mL), streptomycin (100 µg/mL) (Gibco, NY, USA) and incubated at 37 °C under 5% CO_2 and 95% humidity conditions.

Immunohistochemistry (IHC)

Formalin-fixed paraffin-embedded (FFPE) of human normal bladder (National Forensic Organization, Tehran, Iran) and high grade human bladder carcinoma (Imam Khomeini hospital, Tehran, Iran) specimens were cut to a 4 µm of thickness using a microtome instrument and were mounted on positively charged slides. The sections were deparaffinized using xylene, and then dehydrated with decrement concentrations of ethanol. Antigen retrieval was performed by heating the slides at 94 °C for 30 min in citrate buffer (10 mM, pH: 6). After three times washing with Tris-buffered saline (TBS) containing .1% BSA in pH: 7.4 (TBS-BSA), the slides were treated by 3% H_2O_2 (diluted in TBS) for 15 min in dark and at room temperature (RT) to eliminate the endogenous peroxidase activity. Goat serum (5% in 2.5% TBS- BSA) was added to the sides for 30 min for blocking. Anti-FMOD mAb clone 2C2-A1, anti-beta actin, and mouse IgG isotype control antibodies (PadzaCo., Tehran, Iran) (10 µg/mL concentration diluted in 2.5% TBS-BSA) were added to slides for 60 minutes at RT in a humidified chamber followed by three times washing and incubating with EnVision (BioGenex, United States) detection system for 30 min at RT. Afterward, 3, 3'-diaminobenzidine (DAB) chromogen (BioGenex, United States) solution was added and Mayer's hematoxylin (Merck, Darmstadt, Germany) was employed for counterstaining. The sections were extensively washed with deionized water and dehydrated by ethanol in a decremental manner. Finally, the slides were mounted using Entellan (Merck, Darmstadt, Germany) and observed under a fluorescent microscope (Olympus, Tokyo, Japan)[17].

Immunocytochemistry(ICC)

The cells were seeded at a density of 2×10^4 on 8-well coverslips (Germany, Marienfeld GmbH, Lauda-Königshofen) using complete RMPI-1640 medium and incubated overnight at 37 °C in moistened air with 5% CO_2. After overnight incubation, the slides were washed and fixed with cold acetone (at -20 °C) for 2 min following by twice washing with PBS and drying at 4 °C for 30 min. The slides were washed by TBS (pH: 7.4) and TBS-BSA three times (3×3min). In order to prevent the unspecific binding sites, blocking was performed using 10% sheep serum in a 1% TBS-BSA buffer for 30 min at RT. The slides were incubated with 10 µg/mL anti-FMOD mAb or isotype control mAbs diluted in 2.5% TBS-BSA for 60 min at RT. The slides were then washed and re-incubated with FITC-conjugated sheep anti-mouse Ig (PadzaCo., Tehran, Iran) at RT for 45 min. To counterstain the cell nuclei, 1µg/mL DAPI (4`,6-diamidino-2-Phenylindole m) (USA, Calbiochem) was used for 5 min[18]. Finally, the slides were mounted using 50% TBS- glycerol and subjected to a fluorescent microscope (Olympus BX51, Tokyo, Japan).

Flow cytometry analysis

All cell lines were cultured to reach a confluency of 70-80%, harvested by citrate buffer, washed three times using pre-cold phosphate-buffered saline (PBS) and blocked with 5% sheep serum for 30 min at 4 °C. The harvested cells were incubated with 10 µg/mL anti-FMOD mAb or isotype control mAb for one hour at 4 °C followed by washing with pre-cold PBS and

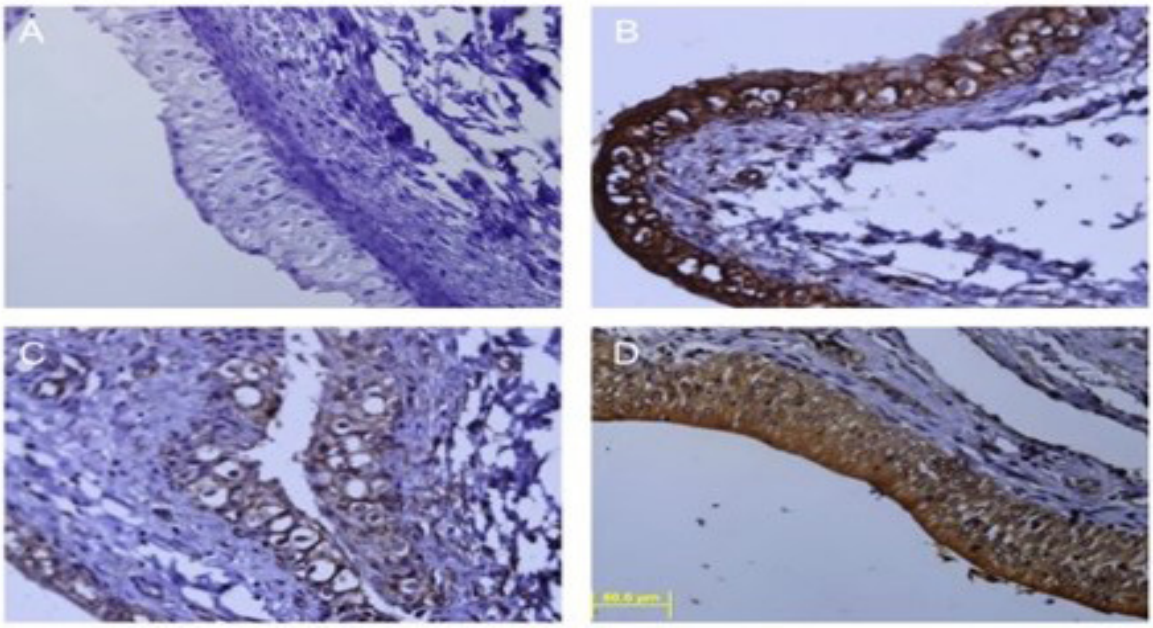

Figure 1. Detection of fibromodulin (FMOD) in formalin-fixed paraffin-embedded bladder cancer and normal tissues by immunohistochemistry (IHC). **A)** Bladder carcinoma tissue stained by mouse IgG isotype control antibody **B)** Bladder carcinoma tissue stained by anti-beta actin antibody C) Normal bladder tissue stained by anti-FMOD murine monoclonal antibody (mAb) clone 2C2-A1 D) Bladder carcinoma tissue anti-FMOD mAb clone 2C2-A1. EnVision detection system (BioGenex, United States) was employed for signal detection and Mayer's hematoxylin was used for counterstaining in all slides (Original magnification, ×50).

incubated with FITC-conjugated sheep anti-mouse Ig (1:50 dilution) for 45 min at 4 °C in a dark place. Finally, the cells were washed with PBS and analyzed using FloMax software (Partec, Nuremberg, Germany) [19]. The average total cell surface expression of FMOD was determined by multiplying of mean fluorescence intensity (MFI) to the percentage of positivity (POP) (MFI×POP)[20].

Cell apoptosis assay

The cells were seeded in a six-well plate ($1x10^6$ /well) and treated with 10 µg/mL anti-FMOD mAb or isotype control mAb for 6 and 12h. The cells were detached and washed for three times using pre-cold PBS and in-

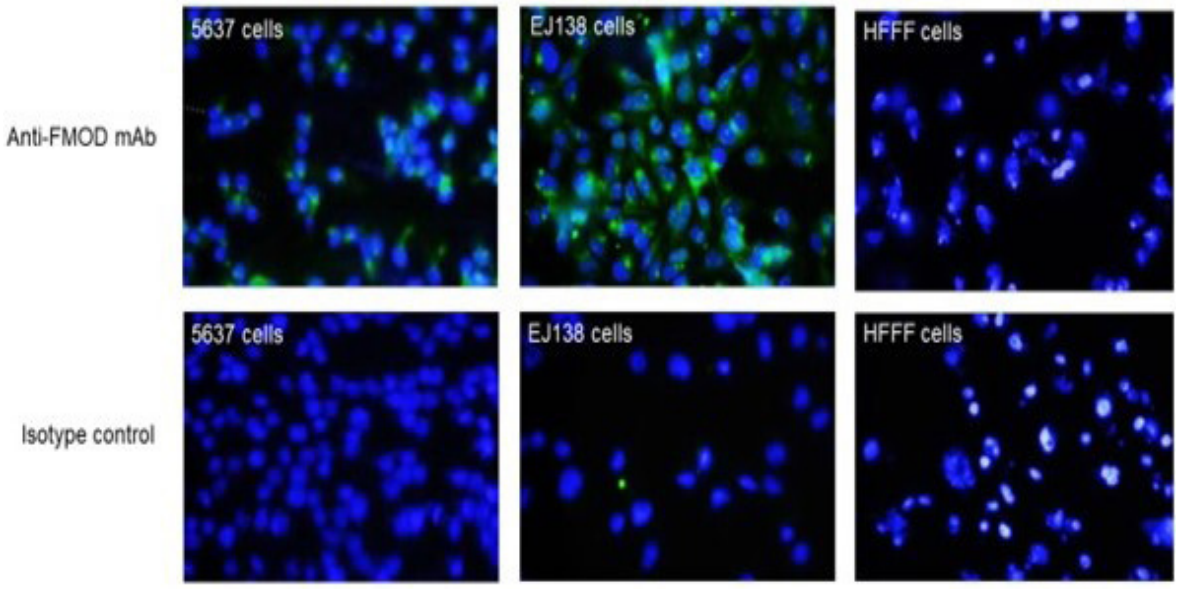

Figure 2. Detection of fibromodulin (FMOD) in bladder carcinoma cell lines by immunocytochemistry (ICC). The Upper panels are 5637, EJ138 and HFFF cells stained by anti-FMOD murine monoclonal antibody (mAb) clone 2C2-A1 and the lower panels are 5637, EJ138 and HFFF cells stained by mouse IgG isotype control antibody as primary antibodies. FITC-conjugated sheep anti-mouse antibody was used as secondary antibody and DAPI was used for counterstaining the nucleus (blue).

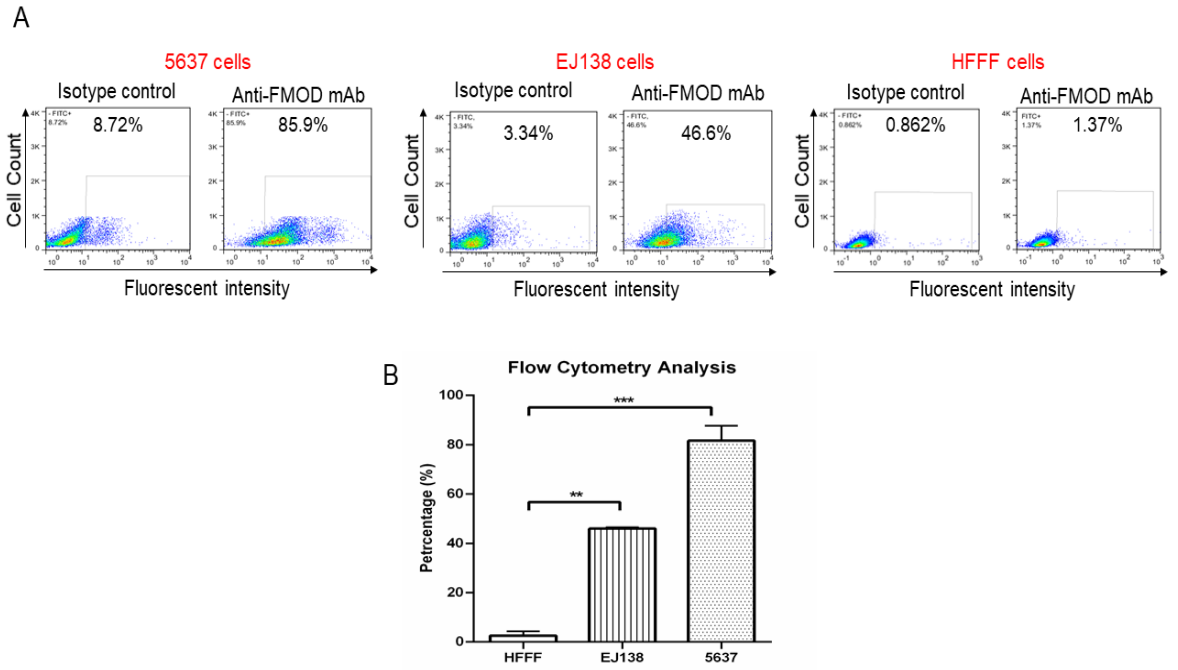

Figure 3. Detection of fibromodulin (FMOD) in bladder carcinoma and normal cell lines using flow cytometry. **A)** Anti-FMOD mAb clone 2C2-A1 could detect FMOD in 85.9 % and 46.6% of 5637, and EJ138 cells as bladder cancer cell lines and 1.30% of HFFF cells as a normal cell. The left diagrams are the obtained values for isotype controls in all three cell lines. **B)** The bar graph of FMOD expression average in 5637, EJ138, and HFFF cells (**: $p \leq .01$; ***: $p \leq .001$)

cubated with 1 µL Annexin V-FITC (BD Biosciences, San Jose, CA) and 2 µL propidium iodide (PI) (BD Biosciences, San Jose, CA) for 15 min at RT in the darkness. The percentage of apoptotic cells as well as live cells were measured using Partec PAS III flow cytometer (Partec GmbH, Germany). The data were analyzed by FlowJo software version 10[21].

Statistical analysis

Statistical analysis was carried out by one-way and two-way ANOVA. The results were illustrated as mean ± SD and p-values less than .05 were considered statistically significant.

RESULTS

Immunohistochemical staining for evaluation of FMOD expression

Immunohistochemistry results of the stained human bladder carcinoma tissues (using anti-FMOD mAb clone 2C2-A1) showed high level of FMOD expression in comparison to the normal bladder tissues. The expression of beta-actin as positive control was observed while no signal detected in isotype control (**Figure 1**).

Detection of FMOD by immunocytochemistry

The immunocytochemistry results in bladder cancer cell lines (5637 and EJ138) and human normal cell line (HFFF) were also demonstrated in **Figure 2**. Two bladder cancer cell lines expressed FMOD, while no signal was detected in normal cell line.

Cell surface FMOD expression by flow cytometry

The average expression of FMOD in two human bladder cancer cell lines was 84.05 ± 1.85% of 5637 and 46.1 ± .4% of EJ138 cells. In contrast, only 2.56 ± 1.26% of HFFF cells showed FMOD expression (negative control). The arbitrary values of mean fluorescent intensity multiply percentage of positivity (MFI × POP) were 3728.06 for 5637, 899.38 for EJ138 and 3.61 for HFFF cells (**Figure 3**) (**Table 1**).

Apoptosis induction by flow cytometry

For 6 hours incubation, the percentage of apoptosis was 12.7 ± 3.1% (early apoptosis) and 7.7 ± 0.3% (late apoptosis) for 5637 cells. EJ138 cells showed a 18.2 ± 9.5% (early apoptosis) and 4.97 ± 2.3% (late apoptosis), while HFFF normal cells showed a 2.51 ± .0.9% (early apoptosis) and .34 ± 0.04 % (late apoptosis). The 12 hours incubation, showed a 6.7 ± 0.01% and 11.8 ± 1.5% for 5637 cells, 9.5 ± 1.4% and 24.05 ± 5.6% for EJ138 cells, and 1.08 ± 0.9% and 3.1 ± 0.05% for HFFF cells, respectively. The isotype control mAb also could not induce significant apoptosis in all examined cells (**Figure 4**).

DISCUSSION

In recent years, the role of ECM components in cancer pathogenesis and their importance in cancer progression, have gained more attention[22,23]. Although, fibromodulin is one of the active proteoglycan of ECM but its pathophysiological role in cancer development and progression is not yet fully understood.

In the present study, the expression of FMOD was assessed with three different readout systems such as IHC, ICC and flow cytometry by anti-FMOD mAb. The immunohistochemistry results revealed a higher FMOD expression in bladder cancer tissues in comparison with normal samples (**Figure 1**). Both bladder cancer cell lines were expressed FMOD in immunocytochemistry experiments, while no signal was detected in human normal HFFF cell (**Figure 2**). Reyes et.al. reported overexpression of FMOD in rat prostate cancer

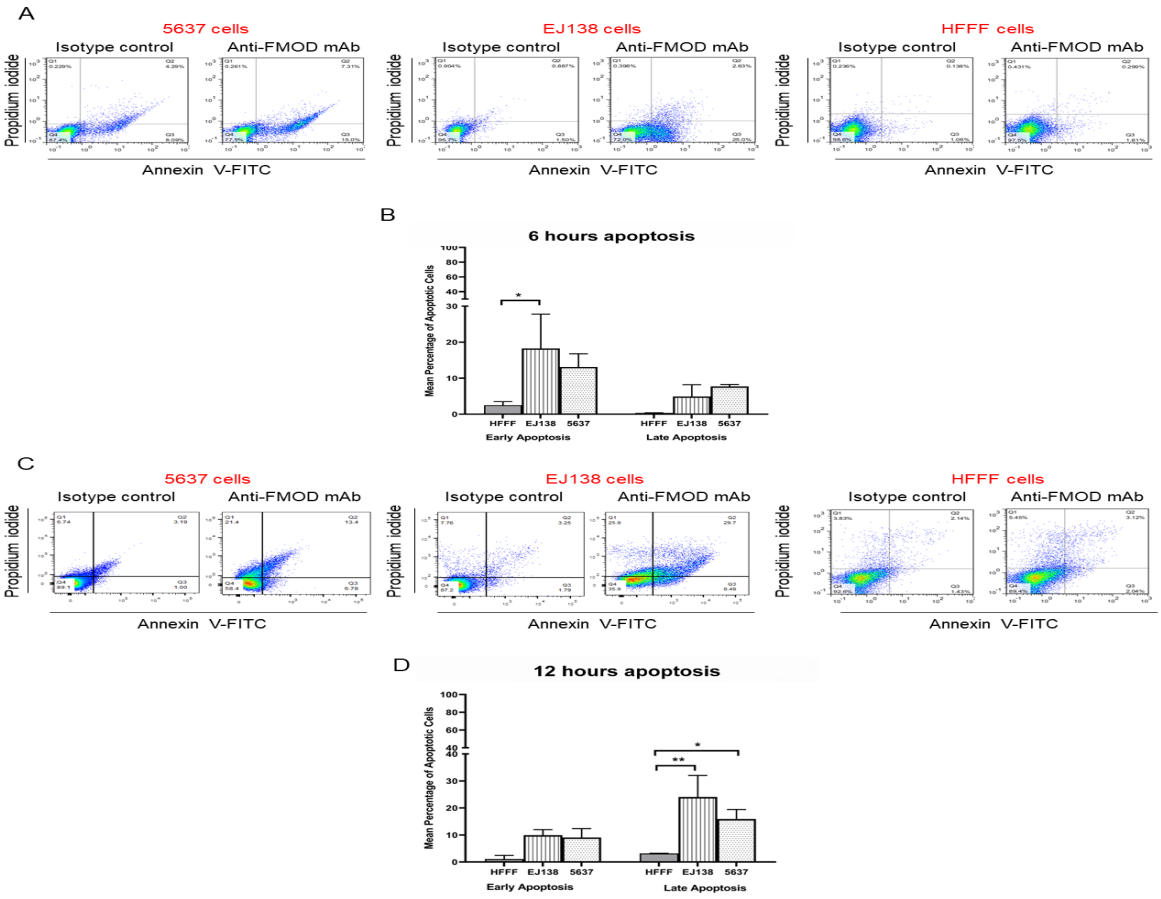

Figure 4. Measurement of apoptosis in bladder carcinoma and normal cell lines using flow cytometry. **A)** Detection of early and late apoptosis after 6 hours of treatment of the cells by anti-FMOD monoclonal antibody (mAb) clone 2C2-A1. The percentage of viable cells after treatment were 77.5 and 72% for 5637 and EJ138 (as bladder cancer cell lines) respectively and 97.5% for HFFF cells. **B)** The bar graph of apoptosis induction for 6 hours incubation by anti-FMOD mAb clone 2C2-A1. **C)** The same experiment after 12 hours treatment, the percentage of viable cells were reduced to 58.4% in 5637 and 35.9% in EJ138 cells, the viable cells percentage for HFFF was same to the result of 6 hours treatment. **D)** The bar graph of apoptosis induction for 12 hours incubation by anti-FMOD mAb clone 2C2-A1. The mouse IgG isotype control mAb did not induce apoptosis in the examined cells also the anti-FMOD antibody could did not induce apoptosis in HFFF cells as a normal cell line (*: $p \leq .05$; **: $p \leq .01$).

cell lines by microarray at transcript level[24]. The expression of FMOD has also been shown in human prostate cancer cells at transcript and protein levels [25]. The aberrant expression of FMOD in different cancers such as B-CLL[11,12], prostate cancer[13], and glioblastoma[14] has also been reported. In our study, such overexpression of FMOD was detected in bladder cell lines and tissues from primary bladder carcinoma patients. This probably marks FMOD as a tumor-associated marker [25].

The flow cytometry results revealed that 2C2-A1 mAb could detect cell surface fibromodulin in 84.05 ± 1.85% and 46.1 ± .4% of 5637 and EJ138 bladder cancer cells, respectively (**Figure 3**). The differences in FMOD expression level might be related to the origin and nature of the cells. The 5637 cell line is known as a non-invasive grade II While the EJ138 cells is considered as an invasive transitional bladder carcinoma[26]. Lower expression of FMOD in EJ138 is associated to an invasive phenotype of bladder cancer which could be used as a differential marker in bladder carcinoma grading. To validate the high level expression of FMOD and its relationship to invasiveness of bladder carcinoma, an extended study on a large group of patients is necessary. Retrospective studies have reported that FMOD is a cytosolic or secretory protein especially in normal cells [12] with no cell surface expression. Here, we revealed that FMOD is not only localized to cytoplasm environment, but also expressed on cell surface[9].

In flow cytometry assays, for obtaining an arbitrary value and estimating the average number of receptors on both cell lines, MFI was multiplied to the percentage of reactivity[20]. By using this arbitrary value one may discriminate between cancer and inflammation conditions as there are always trace amount of protein expression in inflammatory conditions compare to higher expression in cancerous cells.

Our findings revealed significant apoptosis induction in both bladder cancer cells upon treatment with 2C2-A1 mAb (**Figure 4**). The high percentage of apoptosis in both bladder cell lines and neglectable apoptosis induction in normal HFFF cells might indicate FMOD as a survival factor.

FMOD modulates TGF-β functions such as apoptosis

through its binding site inhibiting the role of TGF-β in apoptosis induction[10]. Apparently, anti-FMOD antibody blocks the binding site of FMOD to TGF-β which subsequently increases apoptosis.

Interestingly, in vitro apoptosis induction by anti-FMOD, occurred in the lack of any immune system mediators such as antibody dependent cell mediated cytotoxicity (ADCC) and complement dependent cell cytotoxicity (CDC). Therefore, we speculate that using anti-FMOD antibody as anti-cancer agent strengthens the property of anti-FMOD antibody.

Another output from current study might be the use of anti-FMOD antibody as a diagnostic tool for immunoassay-based detection and characterization of bladder cancer cells. GPI- anchored phenomenon might expand this hypothesis to other secreted proteoglycans such as PRELP, decorin, biglycan, lumican, keratocan, and osteoadherin[9]. It is wise to study the cell surface expression of other proteoglycans in bladder carcinoma.

Common strategies for treatment of bladder cancer such as surgery, radiation therapy, intravesical chemotherapy such as mitomycin C and intravesical immunotherapy like bacillus Calmette-Guerin (BCG)[27] have side effects on cancer targeted therapy. Consequently, targeting by therapeutic agents especially monoclonal antibodies might be considered as an option for treatment of bladder cancer.

The current diagnostic methods for bladder carcinoma include an invasive method such as cystoscopy and biopsy and non-invasive method like urine cytology, CT-sacn and MRI[28].

Several markers like UroVysion, NMP22, BTA and ImmunoCyt/uCyt+ are also used for detection of bladder carcinoma. Although these tests are highly sensitive and specific, they are expensive[29]. Using specific monoclonal antibodies reduces many of such complications. Therefore, a noninvasive diagnostic as well as a monitoring technique using immunohistochemistry and urine samples from patients with bladder cancer using anti-FMOD antibody in flow cytometry technique is recommended.

This study indicates the critical role of FMOD in bladder cancer cell surviving, therefore could be applied as a valuable target in bladder cancer therapeutics. The results from this part are in line with our previously reports emphasizing usefulness of FMOD targeting by monoclonal antibody and silencing its gene using siRNA as a cancer therapy strategy in CLL[9,30].

The cell surface expression of FMOD and survival dependency of bladder cancer cells to its signaling pathway, suggest FMOD as a promising target for cancer treatment by monoclonal antibodies.

CONCLUSIONS

Taken together, our findings showed high level cell surface expression of FMOD in bladder cancer cells and tissues. Therefore, anti-FMOD antibody might further assist among other current diagnostic tools for bladder cancer cell. Furthermore, targeting FMOD by monoclonal antibodies might be considered as a combinatorial strategy for treatment of bladder cancer.

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CONFLICT OF INTEREST

There is no conflict of interest in this work.

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