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ORIGINAL ARTICLE

GPI-Anchored Fibromodulin as a Novel Target in Chronic Lymphocytic Leukemia: Diagnostic and Therapeutic Implications

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ABSTRACT

Background: We have previously reported the aberrant expression of Fibromodulin (FMOD) in patients with chronic lymphocytic leukemia (CLL). Although FMOD has been considered as a cytoplasmic or secretory protein, we discovered the cell surface expression of FMOD in leukemic B cells via anchoring with glycosylphosphatidylinositol (GPI). **Objective:** To evaluate FMOD as a new biomarker in CLL patients in comparison with healthy individuals. **Methods:** A monoclonal antibody was generated against human FMOD. The cell surface expression of FMOD in 52 CLL patients and 45 healthy individuals were compared by flow cytometry. A bacterial phosphatidylinositol-specific phospholipase C (PI-PLC) was used to determine the cell surface localization of FMOD using ELISA and flow cytometry techniques. Annexin V-FITC and propidium iodide (PI) was used to detect apoptosis induction in CLL PBMCs following *in vitro* incubation with anti-FMOD mAb. **Results:** The results demonstrated the widespread cell surface expression of GPI-anchored FMOD in CLL patients (median: 79.9 %), although healthy individuals had low FMOD expression (median: 6.2 %) ($p \leq 0.0001$). The cut-off value of FMOD expression was estimated with high sensitivity and specificity at 17.9%. Furthermore, *in vitro* apoptosis induction of leukemic cells following incubation with anti-FMOD mAb showed a direct apoptosis of CLL cells (27.9%) with very low effect on healthy PBMCs (6%). **Conclusion:** The membrane-anchoring of FMOD by means of a GPI moiety in leukemic cells supports FMOD as a highly potential diagnostic and therapeutic target in CLL patients.

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INTRODUCTION

Chronic lymphocytic leukemia (CLL) is typically composed of a population of small, round, and mature B-lymphocytes in blood, co-expressing CD19, CD5 and CD23 and a weak expression of CD20, CD79b, and surface immunoglobulin (1). Flow cytometry with immunophenotyping has corroborated the diagnosis of CLL in most patients (2). However, CLL is a heterogeneous disease manifested as a highly variable clinical course. Once the diagnosis of CLL is confirmed, patients should undergo extra laboratory evaluation and staging to aid the physician in prognosis and treatment (3). Due to the heterogeneity and complexity of CLL cells, it is of necessity to discover novel biomarkers to enhance diagnosis accuracy and gain insights into more effective treatment. Fibromodulin (FMOD) is a member of small leucine-rich proteoglycan (SLRP) family, classified as a biologically active component of the extracellular matrix. In addition to four other SLRP members, namely lumican, proline arginine-rich end leucine-rich repeat protein (PRELP), keratocan, and osteoadherin, FMOD belongs to class II of SLRP family. The SLRP members conduce to the vital functions of biological and pathophysiological processes, including collagen fibrillogenesis, matrix assembly, signal transduction and tumor growth (4). FMOD protein (42-80 kDa) exhibits a wide tissue distribution, with the highest abundance in articular cartilage, tendon, and ligament (5,6). Retrospective studies have reported that FMOD is a cytosolic or secretory protein with no cell surface localization. Here, we showed that FMOD is not merely localized to cytoplasm or extracellular environment, but also localized and attached onto the cell membrane of leukemic cells via glycosylphosphatidylinositol (GPI) anchoring. GPI anchor is a preformed and highly conserved structure attached by a transamidase to the C terminal of the nascent protein in the endoplasmic reticulum (ER) (7,8). It consists of a lipid moiety with a glycan backbone core regulating a wide variety of biological roles, such as signal transduction, cell–cell interactions, cell adhesion, and host defense through transporting GPI-anchored proteins to cell membrane (9-11). The attachment of GPI-anchored protein to the membrane is via the lipid portion of anchor, and is completely different from transmembrane proteins localized through transmembrane spanning motif in their structure (12). Approximately, 1-2% of the translated proteins in mammals are predicted to be modified by GPI transmidase to attain a GPI anchor which grounds their localization to cell surface (13). Concerning the role of FMOD in cancer pathogenesis, growing evidence has underscored the fact that FMOD is responsible for cancer development and progression. Preliminary results in our previous study showed that FMOD was aberrantly expressed in patients with B CLL and mantle cell lymphoma (MCL) (14). There, FMOD was not detected on the cell surface of CLL, due to the used antibody which was based on the C-terminal of FMOD protein which probably is not accessible in the form of cell surface FMOD. In this study, we produced a specific mAb against the N-terminal part of FMOD and were able to show that FMOD is localized on the cell surface of CLL via the GPI-anchoring mechanism. Although the rationale behind the aberrant expression of GPI-anchored FMOD in CLL is not clear, the role of FMOD in CLL pathogenesis should not be ignored, which might be supported by the results obtained from *in vitro* apoptosis induction of CLL PBMCs using FMOD targeting by anti-FMOD mAb.

MATERIALS AND METHODS

Ethical Approval. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Patients and Controls. The present study is comprised of 52 untreated CLL patients referred to Firoozgar Hospital (Tehran, Iran) and 45 healthy individuals. Patients were analyzed for biological and clinical characteristics, such as age, gender, Rai stage, white blood cell count, haemoglobin level, platelet count and treatment history as well as CD5, CD19, CD23, CD38 and ZAP70 expression. PBMCs were isolated from peripheral blood samples by density gradient centrifugation using a Ficoll-Paque PLUS (Amersham Biosciences, Uppsala, Sweden) according to the manufacturer's instructions (15). This study was approved by the local ethics committee of Avicenna Research Institute. Patients and healthy individuals were informed as to the content of study and consented to provide sample for research purposes.

Cell Lines. A panel of hematological cancer cell lines containing I83-E95, 232-B4, WA-C3CD5+ (CLL cell lines), RPMI 8226 (myeloma cell line), Jurkat (Acute T-cell leukemia cell line) and LCL (lymphoblastoid cell line) (16) (National Cell Bank of Iran, Tehran, Iran) were cultured in RPMI-1640 (Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco) at 37°C in a humidified incubator with 5% CO_2 atmosphere.

Immunogenic Peptide for mAb Generation. As previously described, a peptide of VFDNATGLLWI derived from N-terminal region of FMOD protein was designed and conjugated to Keyhole limpet hemocyanin (KLH) (Sigma, St. Louis, MO) via glutaraldehyde linker (17). Four BALB/c mice were immunized with peptide-KLH, according to the protocol described previously (18). The mice were immunized in compliance with the international standard animal welfare guidelines (19).

MAb Production. Hybridoma technology (20) was applied for mAb production as described before (21). Hybridoma cells producing anti-FMOD antibody were screened using indirect ELISA by coating 500 ng/well of immunizing FMOD peptide as previously described (17). The positive clones were sub-cloned four times using limiting dilution to obtain stable clones.

Antibody Purification and Subclass Determination. The affinity column was prepared using immunizing peptides coupled with CNBr-activated Sepharose-4B (GE Healthcare, Uppsala, Sweden) according to the manufacturer's instructions. Using affinity chromatography column, antibody purification was performed as described (17). Antibody was classified by use of Iso-Strip mouse monoclonal antibody isotyping kit (Roche, Basel, Switzerland).

Determining Affinity Constant (K_{aff}). The affinity constant (K_{aff}) of the mAb was determined using noncompetitive ELISA according to the method described before (21).

Conjugation of mAb with Horseradish Peroxidase (HRP). To activate the peroxidase, 20 µl of 0.1 M sodium periodate (Sigma) was added to 1 mg of peroxidase in 100 µl of distilled water and was rotated for 20 min at room temperature. The solution was dialyzed against sodium acetate buffer (1 mM, pH 4.4) at 4°C. Subsequently, 2 mg of 2C2 mAb was added to the activated peroxidase solution. The carbonate-bicarbonate buffer was added to the mixture to obtain pH 9 to 9.5 by rotating for 2 h. For blocking, 100 µl of fresh sodium borohydrate mixture (4 mg/ml) (Merck) was added to the solution and

rotated at 4°C for 1.5 h. The mixture was ultimately dialyzed overnight against PBS at 4°C.

Flow Cytometry. Purified PBMCs from 52 CLL patients and 45 healthy individuals in addition to a panel of hematological cell lines were analyzed by 2C2 mAb as a primary antibody following FITC-sheep anti-mouse (Padza) as a probing antibody. The method was previously described in detail (21). Rabbit anti-FMOD pAb (Thermo Fisher Scientific, Rochester, NY) was employed as a positive control antibody following probing with FITC sheep anti-rabbit (Padza).

GPI-anchor Cleavage by Phospholipase C. To confirm that FMOD is a GPI-anchored protein, 3 CLL cells were incubated with phosphatidylinositol-specific phospholipase C (PI-PLC) (5,22). Briefly, 1×10^6 CLL cells were treated with 16 units of PI-PLC (Sigma) in PBS 1X at 37°C for 1 h. Enzyme-treated samples were centrifuged for 5 min at 1500 rpm, where the supernatant was collected. Cell pellets were analyzed in flow cytometry as in the method previously described (21,23), and supernatants were analyzed by ELISA (17). Briefly, 50 µl of supernatant was coated in ELISA plates. Subsequently, 5 µg of anti-FMOD mAb was added. HRP-sheep anti-mouse immunoglobulin (Padza, Iran) was used at a dilution of 1:2000. The enzymatic cleavage of CD73 GPI-anchor protein and CD20 was examined as positive and negative controls, respectively.

Immunoprecipitation (IP). CNBr-activated Sepharose-4B (GE Healthcare) was swelled and activated according to the manufacturer's instructions. Subsequently, 2C2 mAb was coupled with the resin, rotated gently overnight at 4°C, and centrifuged at 27 rcf for 5 sec followed by blocking with 0.1 M Tris-HCl. The conjugated resin was washed with 0.1M Tris-HCl containing 0.5 M NaCl, pH 8, and, subsequently, with sodium acetate containing 0.5 M NaCl, pH 4 (three cycles). CLL or healthy PBMCs cell lysates were added to conjugated resin and incubated overnight at 4°C. After washing the unbound proteins, FMOD was eluted using 0.2 M glycin-HCl, pH 2.5. Finally, the eluted fractions and cell lysates were immunoblotted with Rabbit anti-FMOD pAb (Thermo Fisher Scientific, diluted 1:1000) and HRP-sheep anti-rabbit immunoglobulin (diluted 1:3000).

Western Blot. The preparation of cell lysates and Western blot have already been described elsewhere (17). HRP-conjugated anti-FMOD mAb (diluted 1:350) and biotinylated rabbit anti-FMOD pAb (Lifespan Biosciences) (0.5 µg/ml) were utilized as primary antibodies. HRP-conjugated streptavidin (Thermo Fisher Scientific) was used to probe the biotinylated pAb. To detect β-actin protein as internal control, mild stripping buffer containing 200 mM glycine, 0.1% SDS, and 1% Tween 20, pH 2.2 was used to remove the previously incubated antibodies from the PVDF membrane. Afterwards, PVDF was incubated with 5 µg/ml of anti β-actin mAb (Padza) for 45 min followed by probing with HRP sheep anti-mouse immunoglobulin (1:3000).

Immunofluorescent Staining. Forty thousand cells were seeded on cover slips (Marienfeld GmbH, Lauda-Königshofen, Germany) and fixed using cold acetone. Subsequently, immunofuorescent staining using 2C2 mAb was performed according to the method described previously (17).

Apoptosis Assay. PBMCs were purified from blood samples of CLL patients and healthy individuals under sterile conditions. Four hundred thousand cells were cultured in 400 µl serum free media (Gibco) in 24-well plate for 2 hours, and then treated with 10 µg/ml of 2C2 or irrelevant isotype control mAbs (Padza). Staurosporine (2 µM) (Sigma) was served as positive control. Fully human anti-CD20 mAb (ofatumumab, 10 µg/ml, GlaxoSmithKline, Brentford, UK) was used for comparison. Following 14 to 18 h of incubation, cells were removed from the culture and evaluated for apoptosis by double

staining with Annexin V–FITC and propidium iodide (PI) (BD Biosciences, San Jose, CA) in 1X binding buffer followed by FACS analysis using BD FACSCalibur flow cytometer. In order to calculate the percentage of apoptotic cells, the amount of cell percentages in early (*i.e.* Annexin V^+, PI^-) and late (*i.e.* Annexin V^+, PI^+) stages of apoptosis were added together.

Statistical Analysis. Comparison between two groups was performed by Student's t-test for parametric data and Mann–Whitney U test for nonparametric data. The optimal cut-off value of FMOD expression was determined using receiver operating characteristic (ROC) curve to discriminate patients from healthy individuals. Analyses were performed using GraphPad Prism 6 software and statistical significance was defined as $p \leq 0.05$.

RESULTS

Peptide design and mAb generation.

A 11-mer peptide was selected from the LRR1 domain of FMOD protein and used for mAb production. Among 6 obtained stable hybridoma clones, one clone (2C2) was selected as final developed mAb based on its specificity and target detection for further immunoassay experiments. The subclass of generated mAb was IgG2b with κ light chain. Following the purification of 2C2 mAb with affinity chromatography columns, its immunoreactivity with immunizing peptide was assessed in indirect ELISA (Figure 1). Next, K_{aff} value of 2C2 mAb was determined as 2.6×10^{10} M^{-1}.

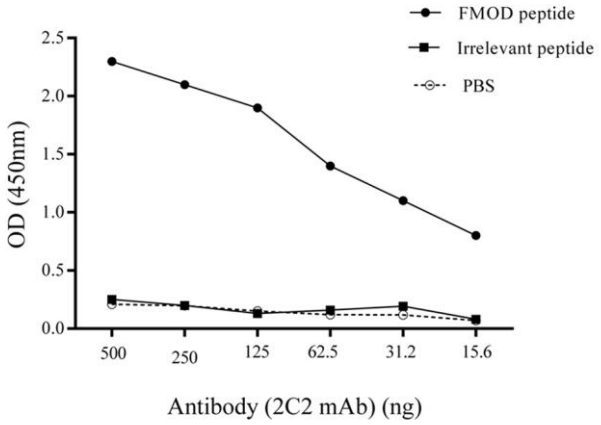

Figure 1. The reactivity of 2C2 mAb with immunizing peptide was assessed in ELISA. FMOD Peptide was coated (500 ng/well) and 2C2 mAb was titrated from 500 ng/well. The specificity of mAb was confirmed by lack of reactivity with an irrelevant peptide and PBS.

FMOD expression in hematological cell lines.

Our previous results showed that FMOD gene was expressed only in CLL and mantle cell lymphoma cell lines (14). So, to examine the expression of FMOD at protein level on the cell surface of CLL cell lines, I83-E95, 232-B4 and WA-C3CD5+ cell lines were investigated. Flow cytometry showed that 2C2 mAb detected FMOD on the surface of CLL cell lines (ranging from 33% to 73%) but not in FMOD non-expressing cell lines including RPMI 8226 (myeloma cell line), Jurkat (Acute T-cell leukemia cell line) and

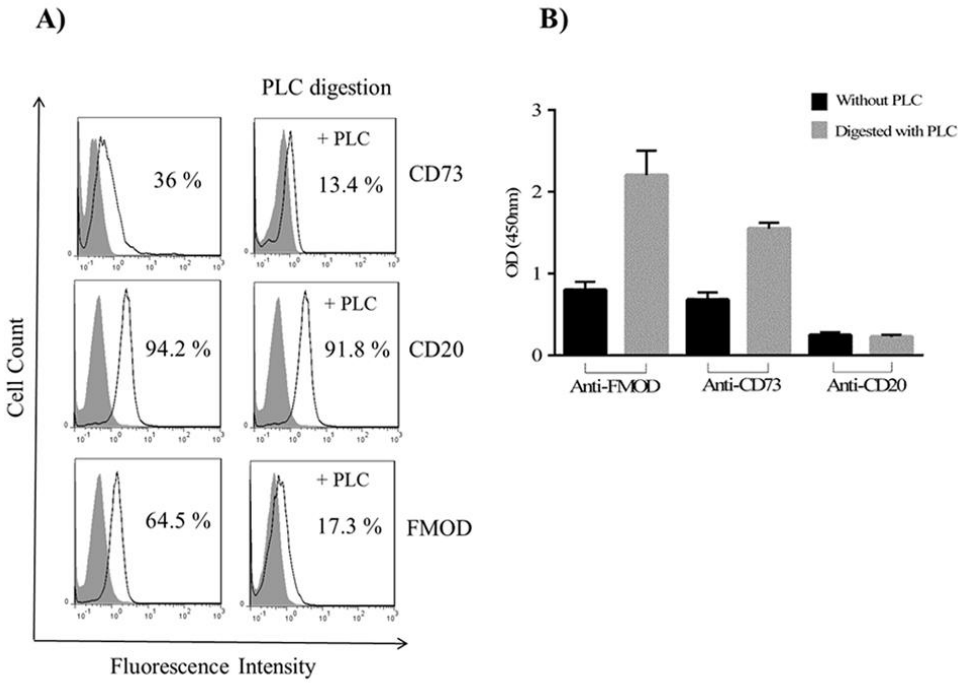

Figure 2. The enzymatic digestion of GPI-anchored FMOD in CLL cells. A) CLL PBMCs were incubated with or without phosphatidylinositol-specific phospholipase C (PI-PLC) and subsequently stained using 2C2 mAb in flow cytometry. Reduction of 73% in surface expression of FMOD was detected in PI-PLC treated cells. The surface expression of CD73 (as positive control) was reduced about 62%. **B)** Detecting soluble FMOD protein following PI-PLC digestion of CLL cells by ELISA. Release of FMOD to the supernatant of PI-PLC treated cells was 1.7 folds more than untreated cells. Obviously, 1.5 folds increase in the release of CD73 (as positive control) to the supernatant of PI-PLC treated cells, and almost no change in detection of CD20 (as negative control) verified the results.

LCL (lymphoblastoid cell line). Immunofluorescent staining confirmed the obtained data (Figure S1B). Investigation of FMOD expression in lysates prepared from hematological malignancy cell lines in Western blotting showed two bands (~55-75 kDa) pertaining to FMOD in CLL cell lines, but not in LCL, RPMI 8226 and Jurkat (Figure S1C). FMOD detection using commercial anti-FMOD pAb confirmed the achieved results, and β-actin was detected as internal control.

Detecting the GPI-anchoring of FMOD.

To investigate whether or not FMOD is a GPI-anchored protein, CLL PBMCs were treated with PI-PLC. The surface expression of FMOD was compared before and after enzymatic digestion in flow cytometry analysis. Results showed that 73% of FMOD expression was diminished on the surface of treated cells following PI-PLC digestion (Figure 2A). As expected, 62% reduction was detected in the cell surface expression of CD73 (as positive control), while no significant reduction was identified in the expression of CD20 (as negative control). ELISA results showed that the release of FMOD in the supernatant of PI-PLC treated cells was 1.7 folds more than untreated cells (Figure 2B). Further observed were 1.5 fold increase in the release of CD73, and no significant change in the level of CD20 in the supernatant of PI-PIC treated cells. Overall, the achieved

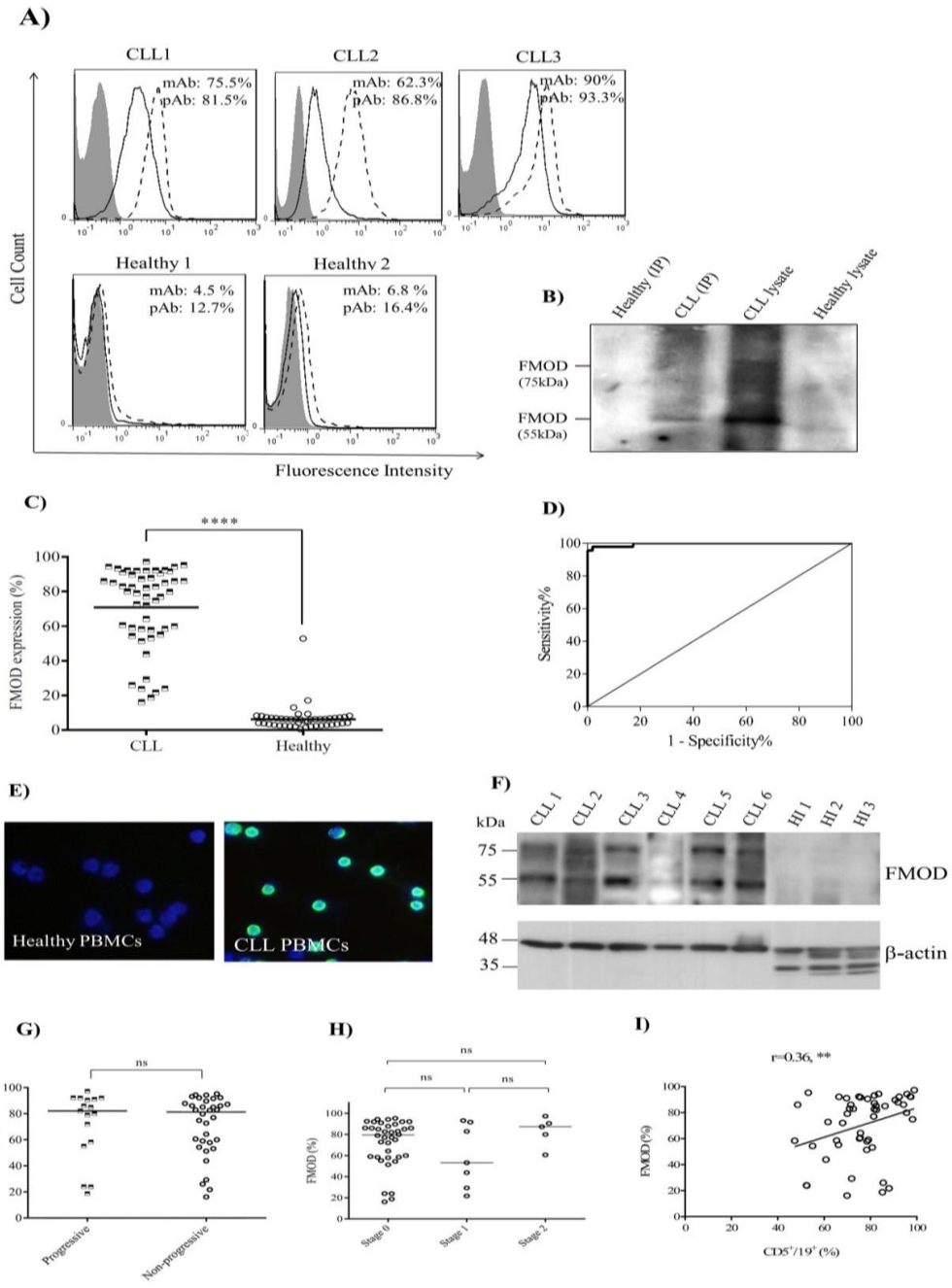

Figure 3. The expression of FMOD in patients with CLL and healthy individuals. A) Detection of FMOD using 2C2 mAb (Solid line) and commercial anti-FMOD pAb (dashed line) in 3 CLL cases and 2 healthy individuals (HI). Gray tinted curve shows detection with isotype control antibodies. **B)** Immunoprecipitation (IP) was performed to confirm the specificity of 2C2 mAb in detecting FMOD. **C)** The expression level of FMOD in 52 CLL patients and 45 healthy individuals (****, P≤0.0001). **D)** ROC curve shows the trade-off between sensitivity and specificity for cut-off value of FMOD expression in CLL and healthy samples. **E)** Immunofluorescent staining showed that 2C2 mAb detected FMOD (green color) in CLL PBMCs, but not in healthy PBMCs. **F)** Western blot showed that 2C2 mAb detected FMOD in CLL cell lysates but almost not in healthy PBMC lysates. **G)** No statistically difference in FMOD expression level was identified in CLL patients with progressive or non-progressive form (p>0.05). **H)** The median expression level of FMOD was not statistically changed in CLL patients with different stages (p>0.05). **I)** The scatter plot demonstrates a significant correlation (**, P≤0.01) between FMOD and CD5+/CD19+ expression levels in PBMCs isolated from CLL patients.

results demonstrated that FMOD was localized on the cell surface of CLL via a GPI anchor.

FMOD expression in CLL patients.

We analyzed the expression of GPI-anchored FMOD on the surface of purified PBMCs from 52 CLL patients which ease the detection of FMOD by flow cytometry as a routine diagnostic test (median age 59 years, range 40-81, 35 males and 17 females) compared to 45 healthy individuals (median age 37.5 years, range 26-70, 19 males and 26 females). The clinical and biological characteristics of CLL patients were summarized in Table 1.

To confirm the specificity of 2C2 mAb in detecting cell surface FMOD by flow cytometry, we applied a commercial anti-FMOD pAb in three CLL patients, and drew a comparison with two healthy individuals (Figure 3A). The expression level of FMOD in CLL cases (CLL1, CLL2, and CLL3) were very similar in both 2C2 mAb (75.5%, 62.3% and 90%, respectively) and commercial pAb (81.5%, 86.8% and 93.3%, respectively). The FMOD expression was very low in healthy individuals, i.e. 4.5% and 6.8% (detected by 2C2 mAb) in comparison with 12.7% and 16.4% (detected by pAb) (Figure 3A). Moreover, immunoprecipitation assay was performed to corroborate the specificity of 2C2 mAb to detect FMOD. Following the capture of FMOD by 2C2 mAb, the corresponding band was detected using rabbit anti-FMOD pAb. Immunoblotting results showed a band of 55 kDa in CLL (IP) fraction and CLL lysate, but not in healthy-IP fraction and healthy PBMCs. (Figure 3B). Detection of FMOD on the surface of CLL and healthy PBMCs using 2C2 mAb demonstrated the widespread and almost high cell surface expression of FMOD in CLL patients (median: 79.9%, 95% CI 64.3% to 86.0%), yet its very low expression in healthy individuals (median: 6.2 %, 95% CI 3.8% to 6.6%) ($P \leq 0.0001$) (Figure 3C). The cut-off value of FMOD expression was estimated at 17.9% (sensitivity: 97.7, specificity: 98.8, AUC: 0.99, $p \leq 0.0001$) (Figure 3D). When cut-off value of FMOD positivity was determined at 17.9%, no patient was identified as negative, and only 1 out of 45 healthy individuals (2.2 %) was introduced as a false positive. Further detected was FMOD expression in CLL patients using Immunofluorescent staining. Figure 3E shows the expression of FMOD in CLL samples in comparison with healthy individuals. Moreover, Western blot analysis was performed to investigate FMOD expression in CLL and healthy PBMCs. Figure 3F represents the expression of FMOD in CLL patients containing non-progressive (CLL 1-3) and progressive (CLL 4-6) forms of disease followed by detection with HRP-conjugated 2C2 mAb in Western blot. Two bands related to ~75 and 55 kDa were detected in CLL patients while a very low FMOD expression in the expected size was detected in healthy individuals (HI 1-3). β-actin was detected as internal control.

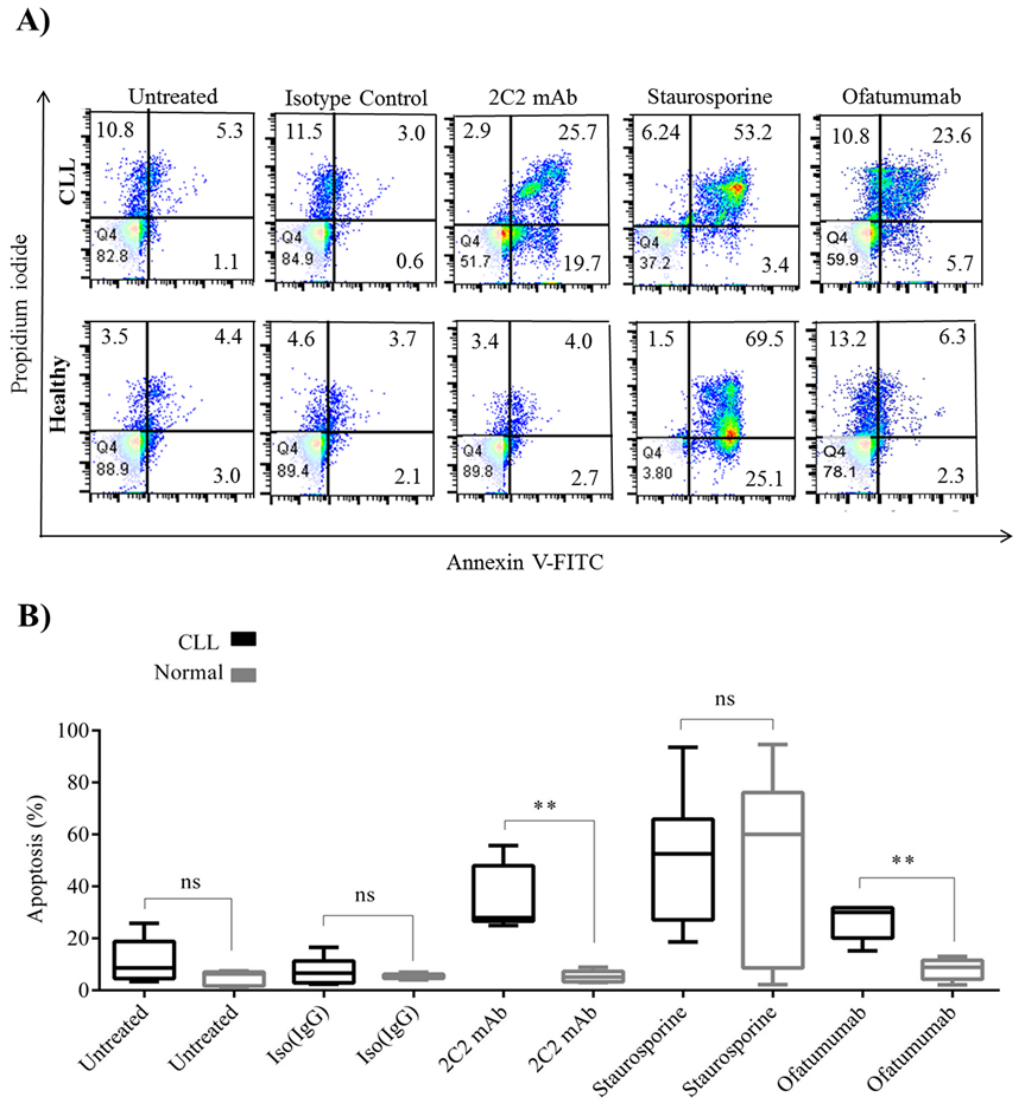

Figure 4. Comparing the apoptosis induction in CLL and healthy PBMCs. A) Dot plot diagram represents apoptosis induction in CLL PBMCs (upper panel) in comparison with healthy PBMCs (lower panel). Cells were treated with 10 µg/ml of 2C2 mAb or irrelevant isotype control mAb as negative control. Staurosporine (2 µM) was served as positive control. Fully human anti-CD20 mAb (ofatumuma, 10 µg/ml) was used for comparison with 2C2 mAb. To calculate the percentage of apoptotic cells, the amounts of cell percentages in early (*i.e.* Annexin V^+, PI^-) and late (*i.e.* Annexin V^+, PI^+) stages of apoptosis were added together. The figure is representative of one from six CLL and six healthy samples. B) Box plot represents the frequency (%) of apoptotic cells induced by 2C2 mAb in CLL patients (n=6) (black color) in comparison with healthy individuals (n=6) (gray color). MAb 2C2 significantly induced apoptosis in CLL PBMCs. Boxes show values between the 25th and 75th percentile values. The horizontal line within the box represents the median value. ns stands for not significant. ** means p≤0.01.

FMOD in CLL patients with different progressions and stages.

All CLL patients, regardless of the progressive or non-progressive form of disease, showed high levels of FMOD expression, with no statistical difference between the two

groups (Figure 3G). We further compared the level of FMOD expression in various stages of CLL where no statistically significant difference was detected (Figure 3H).

Table 1. CLL Patients Characteristics.

Parameter	Value (%)
Sex/ N:52	
Male	N: 35
Female	N: 17
Age at diagnosis/ N:48	
Mean ± SEM	60.33 ±1.56
Median	59
Age range	40-81
Unknown age	N: 4
Treatment history	
Prior treatment	NO
Rai stage/ N:50	
0	N: 38
I	N: 7
II	5
III	0
IV	0
Not known (the data was incomplete)	2
Lymphocyte count (10^9/L)	
Mean ± SEM	41 ± 4.92
Median	30
Range	11-150
Not known (the data was incomplete)	N: 4
$CD5^+/19^+$	
Mean ± SEM	77.13± 1.87
Median	78.65
Range	47.3- 97.4
CD38 (30% cut-off)	
Positive	16
Negative	36
Not tested	0
ZAP 70 (20% cut-off)	
Positive	1

FMOD relationship with other markers.

The relationship between FMOD expression and co-expression of CD5 (T lymphocyte marker) and CD19 (B lymphocyte marker) in B cell population of CLL PBMCs ($CD5^+/CD19^+$) was investigated. FMOD expression was significantly correlated with $CD5^+/CD19^+$ co-expression in PBMCs isolated from CLL patients (r=0.36, p=0.009) (Figure 3I). No significant correlation was observed between FMOD expression and other CLL immunologic markers, such as CD38 and ZAP70 (data not shown), which might be due to the insufficient number of the studied patients.

Apoptosis induction.

To examine the potency of FMOD as a therapeutic biomarker in CLL, we assessed *in vitro* apoptosis induction following the incubation of CLL and healthy PBMCs with 2C2 mAb. Figure 4A shows one from six examined samples in each CLL and healthy individual group. Figure 4B and Table 2 indicate that the treatment of CLL cells with anti-FMOD mAb resulted in 27.9% apoptosis in CLL cells, versus 5.1% apoptosis in healthy individuals ($p \leq 0.01$). Apoptosis induction using 2C2 mAb was specific as no significant apoptosis was induced in CLL cells by isotype control mAb (6.5%). Following the treatment of cells with 2 μM of staurosporine, 52.5% of CLL and 60% of healthy PBMCs underwent apoptosis ($p > 0.05$). Ofatumumab, the fully human anti-CD20 mAb, induced significant apoptosis in CLL cases compared with healthy individuals ($p \leq 0.01$).

Table 2. Percentage of *in vitro* apoptosis induction in CLL and Healthy PBMCs.

Treatment	Apoptosis induction			
	CLL PBMCs		Healthy PBMCs	
	Median (%)	95% CI of Median	Median (%)	95% CI of Median
Untreated	8.6	3.3-25.8	6.3	1-7.4
Iso(IgG) mAb	6.5	2.3-16.5	5.1	4-6.9
2C2 mAb	27.9	24.9-55.7	5.1	3-8.8
Staurosporine	52.5	18.6-93.5	60	2.1-94.6
Ofatumumab	30	15-31.8	8.8	2.1-13

DISCUSSION

FMOD has been shown to be overexpressed in CLL cells using three independent gene expression profiling analyses (24). However, to the best of our knowledge, this is the first study introducing FMOD as a tumor marker, which alongside its secretory variant, can attach to cell membrane via a GPI-lipid anchor. To provide evidence for the claim, we used whole-cell treatment with PI-PLC, as a popular method. Enzymatic digestion of CLL cells resulted in the reduction of FMOD on cell surface following an increase in the supernatant, detected by flow cytometry and ELISA, respectively. PLC digestion was used for the first time in 1963, when it showed a GPI-anchored protein, alkaline phosphatase, was released from mammalian cell surface into the supernatant (25). CD73 was used as a positive control in our experiments, due to the fact that it is anchored to the plasma membrane via a C-terminal serine residue linked to GPI (26). Proteolytic cleavage of CD73 by PI-PLC can release a soluble form of this protein (27). The high expression of CD73 is associated with more aggressive clinical behavior in CLL patients (28). In our study, as it was expected, cell surface signal of CD73 was dramatically reduced in flow cytometry following enzymatic treatment of CLL cells. We further targeted CD20, a regulator of cell-cycle progression of B-lymphocyte, as a GPI-anchored negative control in our experiments. CD20 is a cell surface phosphoprotein with four transmembrane domains and a cytoplasmic termini which restricts its GPI anchoring to the plasma membrane (29). As anticipated, the results of flow cytometry and ELISA did not show

any significant change in the level of CD20 expression, by enzymatic treatment. Our previous study showed that the two other members of SLRP proteins, namely PRELP and opticin were aberrantly expressed in CLL, but not in healthy leukocytes, as it was observed with regards to FMOD (14,30,31). The common extracellular matrix roles of FMOD, PRELP and opticin, as well as their very close gene loci on chromosome 1 (all three in 1q32.1) raise the hypothesis that the gene expressions of these proteins are controlled as a unique gene-cluster reminding epigenetic dysregulation in CLL pathogenesis. Also compared was the level of FMOD expression in CLL with other immunophenotyping markers. A statistically significant relationship was identified when the relationship between FMOD and $CD5^+/CD19^+$ expressions was examined in CLL patients, implying that the aberrant expression of FMOD occurs in B cell population of PBMCs rather than T cells, which, on the other hand, needs further verification. No statistically significant change was seen in the level of FMOD expression in different stages (0, I and II) of CLL. It seems that more comprehensive studies are required to surely declare that FMOD is an adequate factor to be used as a staging marker in CLL patients. Although parameters such as immunoglobulin mutational status or the expression of ZAP70 and CD38 immunophenotyping markers are indicative of the progressive state of CLL, we found no significant difference in FMOD expression between the two progressive and non-progressive cases. This might imply that FMOD expression is a genuine phenomenon of malignant B cells initiating from the formation of cancer cells. We also postulate that FMOD is translocated onto the cell surface exclusively under pathological conditions. To provide evidence for this proposition, we studied the expression of FMOD in an immortalized but not malignant cell line, *i.e.* Epstein-Barr Virus-transformed B lymphoblastoid cell lines, LCL in comparison with leukemic B cell lines including I83-E5, 232-B4 and WA-C3CD5+ (Figure S1). The rather high expression of FMOD on the surface of leukemic B cell lines (33%, 65% and 73.2%) compared with the virtually low expression of FMOD in LCL (8.6%) might imply that FMOD is translocated onto the surface merely in a genuine pathological condition.

Although it is not clear why CLL cells harbor cell surface FMOD, several researchers have suggested that FMOD may undertake vital tasks in the pathophysiology of other disorders. One study showed that the upregulation of FMOD by transforming growth factor beta-1 (TGF-β1) in glioblastoma, promotes cell migration through inducing actin fiber formation (32). Others introduced FMOD as a tumor-associated antigen in primary CLL cells promoting autologous tumor-specific T cell expansion (33). FMOD has been shown to be a potent factor in reprogramming the somatic cells, enabling them to demonstrate pluripotent stem cell features (34). In a similar study, the direct addition of recombinant FMOD protein to cell culture media resulted in the reprogramming of human dermal fibroblasts into multipotent stem cells, generating bone tissues in SCID mice (35). Therefore, it may be suggested that the aberrant expression of FMOD in malignant cells preserve the pluripotency of these cells. By binding to TGF-β, FMOD modulates its function, sequestering it from extracellular matrix (36). TGF-β is a multifunctional cytokine which stimulates apoptosis in normal B cells, while CLL B cells do not respond to the apoptotic effect of this cytokine (37). Such aberrant response to TGF-β is involved in the expansion of the leukemic clone and the accumulation of long-living B cells arrested at an early stage of their programming (38). The decrease or absence of CLL B cells response to the growth-inhibitory effect of TGF-β might be related to the aberrant expression of FMOD in sequestering TGF-β. In this case, the soluble form of TGF-β in the sera of CLL patients is expected to be lower than healthy

individuals. Apoptosis induction in CLL cells by targeting FMOD might be attributed to the significant role of FMOD in B CLL survival (Figure 4A and B). Following the treatment of leukemic cells with anti-FMOD mAb, 27.9% of cells underwent apoptosis, but the rest of the cells remained intact. This might be due to either the low dosage of mAb used in the treatment of CLL cells or the presence of other survival factors in association with FMOD, enabling malignant B cells to escape from apoptosis. Also studied was the potency of anti-FMOD mAb in targeting FMOD in apoptosis induction in comparison with ofatumumab (39). As shown in Figure 4B, treatment with anti-FMOD mAb resulted in significant apoptosis in CLL cells, but not in healthy PBMCs ($p \leq 0.01$), which is similar to the results obtained from ofatumumab. Here, we suggest a combination of chemo- and immunotherapy regimens containing antibodies targeting FMOD which might improve the quality and duration of responses in CLL. Akin to the higher efficacy has been achieved in the clinic from combination of anti-CD20 antibody (rituximab) with fludarabine and cyclophosphamide in treatment of CLL (40). In conclusion, the presence of GPI-anchored proteins such as CD24, CD52 and CD59 (41-43) in association with FMOD on the surface of CLL cells may imply the importance of their functions in this cancer. The question to be answered is the role of GPI anchoring in the pathogenesis of B cell malignancies or solid tumors. Is this a mechanism for survival or is it involved in proliferation? Here, we suggest targeting CLL using anti-FMOD antibody in combination with other GPI-anchored proteins which might provide a novel approach to battling such ilk of hematological malignancy. Nevertheless, targeting FMOD using antibodies in passive immunotherapy has its own disadvantages because FMOD is widely distributed in extracellular matrix of healthy tissues. To overcome this issue, it is essential to produce antibodies that only recognize FMOD in pathological conditions.

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