Original Article

Production and Characterization of a Murine Monoclonal Antibody Against Human Ferritin

Ali Ahmad Bayat [1], Omid Yeganeh [1], Roya Ghods [1], Amir Hassan Zarnani [2], Reza Bahjati Ardekani [3], Ahmad Reza Mahmoudi [1], Jafar Mahmoudian [1], Farzaneh Haghighat-Noutash [1], and Mahmood Jeddi-Tehrani [1*]

1. Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
2. Nanobiotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
3. Reproductive Biotechnology Research Center, Avicenna Research Institute, ACECR, Tehran, Iran

*** Corresponding author:** Mahmood Jeddi-Tehrani, Ph.D., Monoclonal Antibody Research Center, Avicenna Research Institute, ACECR, Tehran, Iran
Tel: +98 21 22432020
Fax: +98 21 22432021
E-mail: mahjed@yahoo.com
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Abstract

Background: Ferritin is an iron storage protein, which plays a key role in iron metabolism. Measurement of ferritin level in serum is one of the most useful indicators of iron status and also a sensitive measurement of iron deficiency. Monoclonal antibodies may be useful as a tool in various aspects of ferritin investigations. In this paper, the production of a murine monoclonal antibody (mAb) against human ferritin was reported.

Methods: Balb/c mice were immunized with purified human ferritin and splenocytes of hyper immunized mice were fused with Sp2/0 myeloma cells. After four times of cloning by limiting dilution, a positive hybridoma (clone: 2F9-C9) was selected by ELISA using human ferritin. Anti-ferritin mAb was purified from culture supernatants by affinity chromatography.

Results: Determination of the antibody affinity for ferritin by ELISA revealed a relatively high affinity (2.34×10^{9} M^{-1}) and the isotype was determined to be IgG2a. The anti-ferritin mAb 2F9-C9 reacted with 79.4% of Hela cells in flow cytometry. The antibody detected a band of 20 *kDa* in K562 cells, murine and human liver lysates, purified ferritin in Western blot and also ferritin in human serum.

Conclusion: This mAb can specifically recognize ferritin and may serve as a component of ferritin diagnostic kit if other requirements of the kit are met.

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Keywords: ELISA, Ferritin, Flow cytometry, Monoclonal antibody, Western blotting

Introduction

Ferritin is a ubiquitous and highly conserved iron storage protein which plays major roles in iron homeostasis such as protective function against toxic effects of iron overload in cells, cytoprotective antioxidant in pancreatic B-cells and endothelial cells, regulation of the expression of globin genes in erythroid cells, suppression of mRNA translation and proliferation of myeloid progenitor cells [1]. This protein (outside diameter 12-13 *nm*, inside diameter 7-8 *nm*) is produced by nearly all living organisms and is found in eukaryotes and prokaryotes [2]. A 450 *kDa* ferritin complex consists of 24 subunits of two types, H (heavy; 21 *kDa*) and L (light; 19 *kDa*) subunits, and can store up to 4500 iron (Fe^{3+}) ions [3,4]. The H-subunit has a relatively acidic electrophoretic dynamism and acts as a ferroxidase that oxidizes iron to (Fe^{3+}) ions, whereas the L-subunit interferes with iron nucleation, mineralization and long term iron storage [2]. Ferritin that is not associated with iron is called apoferritin and subunit combination varies in different tissues [5].

High serum ferritin levels have been reported in patients with elevated iron stores, and low levels are associated with iron deficiency [6]. Recent studies have reported that perturbations in ferritin levels are associated with the progression of tumor cells in breast cancer [7-9] liver, lung, and prostate cancers [10] by which ferritin perturbations directly instigate tumorigenesis and cause malignant phenotype [7,8]. Since the serum of patients with a variety of tumors contains high levels of ferritin [11-13], it seems that the serum ferritin is a good marker for cancer. Therefore, detection of ferritin by mAb as a rapid and sensitive method is necessary [14]. Besides, anti-ferritin mAb can be used for designing diagnostic kits for measurement of ferritin in various biological fluids. Production of such kits seems to be more appropriate for patients than other methods for evaluation of iron stores such as liver biopsy, or bone marrow biopsy [15,16]. This study aimed to produce and characterize a high affinity murine anti human liver ferritin antibody.

Materials and Methods

Immunization procedure

Two Balb/c mice (6-8 weeks old) were immunized with purified human ferritin [17]. Briefly, each mouse was immunized 5 times with 50 *μg* of ferritin every 2 weeks. The first immunization was performed using complete Freund's adjuvant. Incomplete Freund's adjuvant was used for subsequent immunizations. One week after the last immunization, blood was collected by a vertical incision of the tail vein followed by determination of antibody titers by ELISA. Finally, three days before the cell fusion, 20 *μg* of ferritin (without any adjuvant) were injected intravenously [18].

ELISA

Mouse serum titrations and screening of hybridoma supernatants were performed by Enzyme-linked immunosorbent assay (ELISA). The wells of ELISA plate (Nunc, Roskilde, Denmark) were coated with 50 *μl* of ferritin (10 *μg/ml*) dissolved in Phosphate Buffered Saline (PBS) and then incubated at 37 *℃* for 1 *hr* followed by overnight incubation at 4 *℃*. Then the plates were washed 3 times with PBS containing 0.05% Tween 20 (PBS-T) for 3 *min*. followed by blocking with 2.5% bovine serum albumin (BSA) at 37 *℃* for 1 *hr*. Wells were then washed 3 times and mouse sera (in two-fold serial dilutions starting from 1:500) were added and incubated for 1 *hr* and wells were again washed with PBS-T. Rabbit anti mouse Ig conjugated to horseradish peroxides (1:1000) (Avicenna Research Institute, Tehran, Iran) were added to the wells and incubated for 1 *hr* at 37 *℃*. After washing, 50 *μl* of tetramethylbenzidine (TMB) (Sigma-Aldrich, Missouri, USA) substrate was added to each well and the plates were incubated at room temperature in the dark. After 15 *min*, the reaction stopped by adding 15 *μl* of stop solution (20% H_2SO_4) to each well. The Optical Density (OD) of the reactions was measured at 450 *nm* by an ELISA reader (BioTek, Winooski, VT, USA). The mouse with higher titer of antibody was selected for fusion. To screen the antibody production of hybridoma cells, the same method was done on the cell supernatants.

Hybridoma cell production

Mouse myeloma Sp2/0 cells, used as fusion partners, were cultured and propagated in RPMI-1640 culture medium (Gibco, Gran Island, NY, USA) and 10% Fetal Bovine Serum (FBS) (GIBCO Invitrogen, USA). Spleen cells from the immunized mouse were mixed with the Sp2/0 cells at a ratio of 1:5 (1 Sp2/0 and 5 spleen cells). The mixture was washed twice with pre-warmed RPMI-1640 (37 *℃*). Then, pre-warmed 50% polyethylene glycol (PEG) 1500 (Sigma-Aldrich, Germany) was used for fusion. Selective HAT medium

(Sigma-Aldrich, Germany) was then used for selection of hybridoma cells. The reactivity of culture supernatants was then tested by ELISA [18]. Finally, positive hybridomas were cloned by limiting dilution process [19].

Antibody purification

Anti-ferritin mAb 2F9-C9 was purified from culture supernatants by affinity chromatography using a Hi-Trap protein G column (GE Healthcare, Uppsala, Sweden). Briefly, culture supernatants were filtered through 0.45 *μm* filters and pH was adjusted to 7.5. The elution was performed using Glycine-HCl (0.1 *M*, pH=2.7). The eluted antibody was dialyzed against PBS at pH=7.5 and the reactivity of the purified antibody was determined by ELISA method as mentioned above.

Isotype determination

Goat anti mouse IgG_1, IgG2a, IgG2b, IgG3, IgA and IgM (Sigma-Aldrich, Missouri, USA) at 1/1000 dilution were coated in the wells of ELISA plate (Nunc). Supernatant of the growing hybridoma 2F9-C9 was added to each well. The isotype of 2F9-C9 mAb was then determined according to the ELISA method described above.

Detection of ferritin in human serum with anti-ferritin 2F9-C9 mAb by direct sandwich ELISA

To determine the reactivity of 2F9-C9 with free ferritin and ferritin in human sera, 2F9-C9 mAb (50 *μl/well*, 5 *μg/ml*) was coated in a 96 well plate and then, different dilutions of ferritin from 0 to 250 *ng/ml* as well as human sera (1:5 dilution) were added (50 *μl/well*). HRP-conjugated anti-ferritin polyclonal antibody (Avicenna Research Institute, Tehran, Iran) was then added. After washing, TMB was added as substrate and the test continued as described in ELISA section. For comparison, 1:5 diluted human sera were also tested by a commercial ferritin kit (RADIM, Florence, Calenzano, Italy).

Flow cytometry

Hela (Human cervical carcinoma) cells were harvested by 0.5% trypsin and 0.1% EDTA (Gibco) and permeabilized by permeabilizing solution (Becton Dickinson, USA). Cells were then blocked with 5% sheep serum for 10 *min*, and then cells were incubated with 100 *μl* of 2F9-C9 mAb (10 *μg/ml*) for 1.5 *hr* at 4^oC. Anti-HIV Env (Avicenna Research Institute, Tehran, Iran) with IgG1/K isotype was used as an isotype control [20]. After 3 times of washing with cold PBS, FITC-conjugated sheep anti-mouse Ig (Avicenna Research Institute, Tehran, Iran) (1:50) was added to cells and incubated for 45 *min* in the dark at 4^oC. Cells were washed and sorted by flow cytometry. The data was analyzed using Flowmax software (Partec, Nuremberg, Germany).

Western blotting

Lysates of the cell lines Raji (human burkitt's lymphoma), PC3 (human prostate cancer), K-562 (human myelogenous leukemia), human and mouse liver lysates (lysis buffer: 150 *mM* NaCl, 1 *mM* EDTA, 50 *mM* Tris HCl pH=7.4, 1% Triton X-100, 1% Sodium deoxycholate, 0.1% SDS), supplemented PI (phosphatase inhibitor) (Roche, Basel, Switzerland) and 1% PIC (protease inhibitor cocktail) (Sigma-Aldrich, Missouri, USA) were prepared. The protein concentrations of the lysates were measured by BCA protein assay kit (Thermo Scientific, Rockford, IL. USA). Twenty *μg* of cell lysates and 200 *ng* purified ferritin were run on a 15% SDS–PAGE gel.

After electrophoresis, resolved proteins were transferred onto PVDF membranes (Millipore Corporation, Billerica, Mass chusetts, USA). The membranes were blocked with 5% non-fat milk in PBS-T overnight at 4 ℃. After gentle washing with PBS-T, 2F9-C9 mAb (5 *μg/ml*) was added to the membrane and incubated for 1.5 *hr* at room temperature. The membrane was washed extensively with PBS-T and incubated with HRP-conjugated Rabbit anti-mouse Ig (Avicenna Research Institute, Tehran, Iran) (1:2500) for 1 *hr* at room temperature followed by washing and developing with ECL Chemiluminescence detection system (GE Healthcare).

For validation of protein band specificity detected in Western blot, reactivity of anti-ferritin mAb was blocked with a saturating concentration of ferritin (30:1 ferritin to antibody molar ratio). In this regard, ferritin was added to 2F9-C9 mAb for 1 *hr* at 37 *°C* and then the mixture was added to PVDF membrane. Unblocked anti-ferritin mAb was added to another PVDF as a positive control. The incubations, washings and development of bands were performed as the above [21].

Determination of affinity constant (K_{aff})

The affinity constant (K_{aff}) of 2F9-C9 mAb was determined by ELISA [21,22]. Briefly, different concentrations of ferritin (5000, 2500, 1250, 625, 312.5, 156, 78 and 39 *ng/ml*) were coated in 96 well ELISA plates. Serial dilutions of 2F9-C9 mAb (5000, 1250, 312.5, 78, and 19.5 *ng/ml*) were added to each coated well. Sigmoid curves were plotted using the OD values obtained for different concentrations of mAb. The affinity constant was measured as described elsewhere [23].

Results

Production of anti-ferritin mAb

After immunization of mice with ferritin, the titers of anti-ferritin antibodies in the mice sera were measured by ELISA. Results showed that mouse 1 had higher titer of anti-ferritin Ab (Figure 1). After fusion between splenocytes of mouse 1 and Sp2/0 cells, supernatants of growing hybridoma cells were screened based on reactivity with ferritin by ELISA. In this screening assay, among 20 positive clones, a clone was characterized as 2F9-C9 with high reactivity with ferritin (OD: 1.438). The isotype of 2F9-C9 mAb was determined to be IgG2a (Figure 2) and its affinity was calculated to be 2.34×10^{9} M^{-1} (Figure 3) and (Table 1).

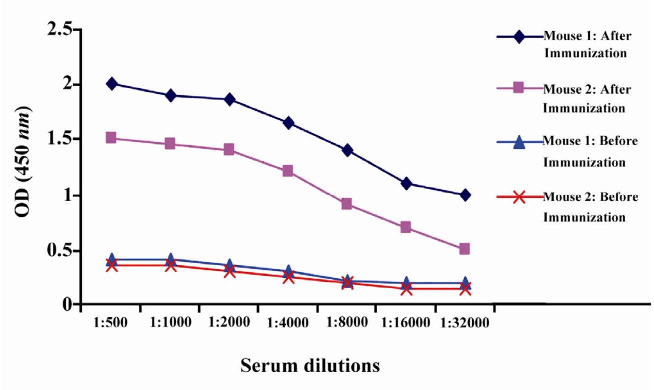

Figure 1. Serum titration of two immunized Balb/c mice by ELISA. Mice were immunized five times by intraperitoneal injection of 50 *μg* human ferritin

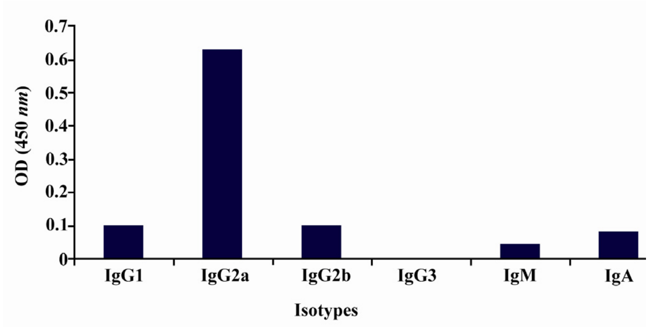

Figure 2. Isotype determination of 2F9-C9 mAb by ELISA

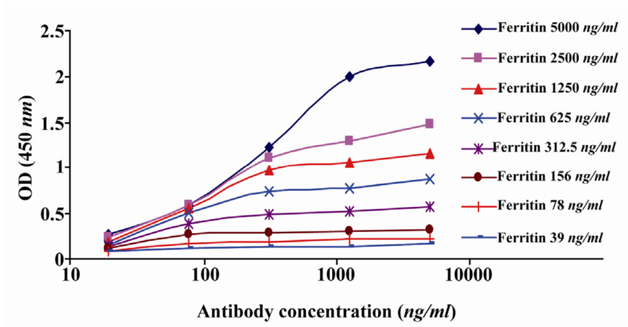

Figure 3. Determination of affinity constant of 2F9-C9 mAb (K_{aff}) by ELISA. Different concentrations of 2F9-C9 were tested against serial dilutions of human ferritin and Kaff was calculated

Table 1. Calculation of 2F9-C9 affinity constant

mAb	[Ag] (*ng/ml*)	OD-50	[Ab] at OD-50 (*ng/ml*)	Kaff (M^{-1})	Avg Kaff (M^{-1})
2C9-F9					
	5000	1.1	240	1.66×10^{9}	2.34×10^{9}
	2500	0.75	125	3×10^{9}	
	1250	0.57	85	2.37×10^{9}	
	625	0.45	55		

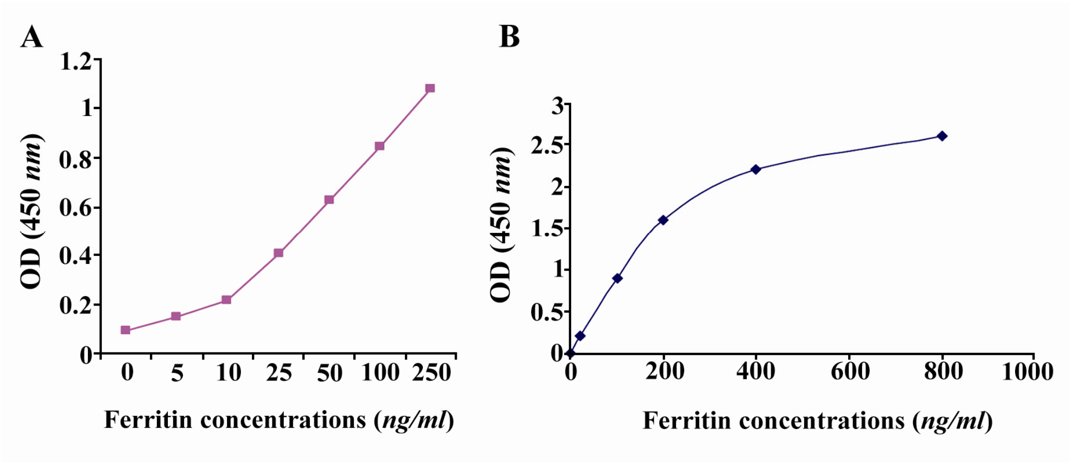

Figure 4. A) In house developed ELISA standard curve for 2F9-C9 mAb; B) Standard curve of commercial kit

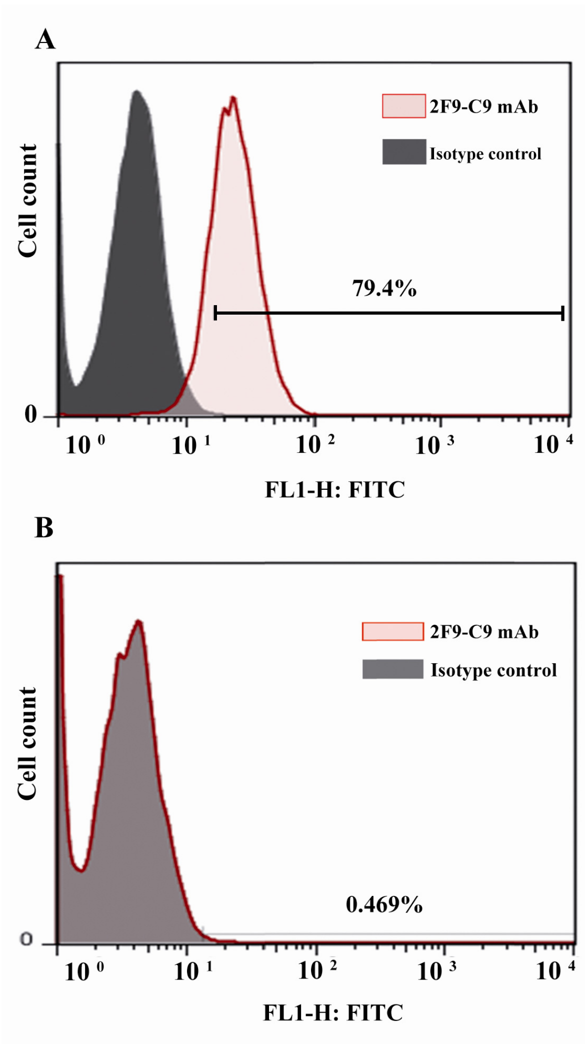

Figure 5. Flow cytometric analysis of intracellular ferritin expression in Hela; A) and HepG2; B) cells using 2F9-C9 mab

Table 2. Determination of ferritin concentration in 5 human serum samples by in house designed ELISA using 2F9-C9 mAb compared with a commercial ferritin measurement kit

Samples	2F9-C9 ELISA	Commercial kit
1	43 *ng/ml*	41.8 *ng/ml*
2	15 *ng/ml*	15.8 *ng/ml*
3	25 *ng/ml*	28.7 *ng/ml*
4	12 *ng/ml*	12.1 *ng/ml*
5	125 *ng/ml*	118 *ng/ml*

Detection of ferritin in human serum

Known concentrations of ferritin were used to plot ELISA standard curve for 2F9-C9 mAb (Figure 4A). ELISA standard curve for the commercial ferritin measurement kit was also plotted according to the manufacturer's instructions (Figure 4B). As shown in table 2, the concentrations of ferritin in 5 human sera which were measured with our ELISA test using 2F9-C9 mAb and the commercial kit were found to be very similar.

Flow cytometric properties of 2F9-C9 mAb

Since Hela cell line expresses ferritin intracellularly, 2F9-C9 mAb was applied for intracellular staining of ferritin in this cell line. Figure 5A shows that 2F9-C9 mAb recognized intracellular ferritin molecules in 79.4% of Hela cells, while the ferritin negative HepG2 cell line showed no ferritin expression by flow cytometry using 2F9-C9 (Figure 5B).

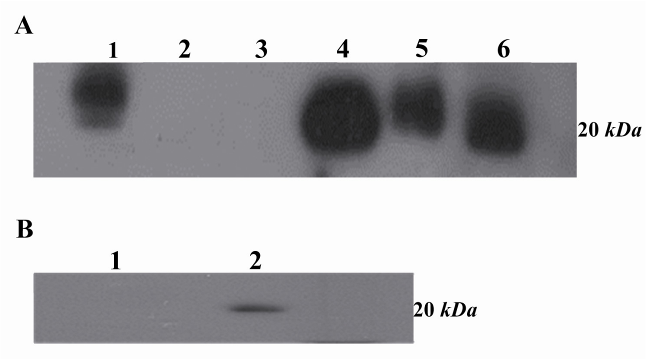

Figure 6. A) Western blot analysis of 2F9-C9 mAb reactivity with different cell lysates and purified ferritin. Lanes 1 and 4 represent human and murine liver lysates, respectively; lanes 2 and 3 include Raji and PC3 cell lysates as negative controls, respectively. Lane 5 includes K-562 cell lysate and lane 6 includes purified human ferritin; B) Lane 1 represents inhibition of 2F9-C9 reactivity with ferritin by saturating amounts of exogenous ferritin. Lane 2 represents unblocked 2F9-C9 reactivity with purified human ferritin

Western blot analysis

Western blot analysis demonstrated that 2F9-C9 mAb recognized ferritin as an approximately 20 *kDa* protein band in human liver, mouse liver and K-562 cells (Figure 6A). Additionally, blocking of the antibody with human ferritin resulted in abrogation of its reacting, hence no band was detected in western blot (Figure 6B).

Discussion

Producing a high affinity monoclonal antibody against human ferritin provides a convenient means for monitoring and tracking ferritin in human body fluids. In this study, a murine anti-ferritin mAb with a high affinity constant was produced and characterized. 2F9-C9 mAb recognizes a specific protein band around 20 *kDa* in Western blot and blocking of the antibody with purified human ferritin led to disappearance of the protein band, confirming the specificity of 2F9-C9 mAb for ferritin. On the other hand, 2F9-C9 mAb recognized intracellular ferritin molecules in 79.4% of Hela cells whereas HepG2 cells were negative. Besides, mAb identified ferritin in some human serum samples in a similar manner to a commercial ferritin measurement kit that also confirms mAb specificity for ferritin. In addition, human serum ferritin contains nearly only L subunits [24] and considering the fact that H and L chains of ferritin have about 60% amino acid sequence homology [3,25,26], 2F9-C9 mAb probably recognizes the L chain or a common epitope on the L and H chains. Human and mouse ferritin have 93% homology in theprotein sequences (NCBI, blastp). Considering the high degree of homology between human and mouse ferritin molecules, it is most probable that the antibody recognizes a shared epitope on both molecules.

To compare with other studies, Lee *et al* reported that only one out of more than 1000 hybrid clones had a high affinity (1×10^{10} M^{-1}) for human ferritin and by using this antibody they showed that under identical conditions, purified ferritin from human spleen and human heart showed 50 and 30% cross-reactivity, respectively [14]. Mel'nikova *et al* also obtained three mAbs with affinity constants ranging from 6×10^{8} to 3×10^{9} M^{-1}. They used these mAbs to define their binding sites on a ferritin molecule. They concluded that only four IgG molecules could simultaneously bind a ferritin molecule [27]. In addition, Nozawa *et al* developed an anti human placental ferritin mAb which was used to measure serum ferritin in three groups: normal women, normal pregnant women and women with gynecological cancers. They concluded that ferritin was a good marker for cancer research [28]. Importantly, ferritin measurement has been applied to study some types of cancers. In this regard, Uesaka produced an anti human liver ferritin that was used to show that serum ferritin levels significantly increased in pancreatic cancer and hepatoma compared to that in normal sera [12].

Conclusion

In conclusion, anti human ferritin mAb 2F9-C9 was shown to react with a 20 *kDa* ferritin subunit. The IgG2a antibody was also shown to be capable of detecting serum ferritin by ELISA and intracellular ferritin by flow cytometry.

Acknowledgement

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Conflict of Interest

The authors have no conflicts of interest to declare.

References

1. Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, et al. Ferritin: a cytoprotective antioxidant strategem of endothelium. J Biol Chem 1992;267(25):18148-18153.
2. Goswami B, Tayal D, Mallika V. Ferritin: A multidimensional bio marker. The Internet Journal of Laboratory Medicine 2009;3(2):http://www.ispub.
3. Theil EC. Ferritin: structure, gene regulation, and cellular function in animals, plants, and microorganisms. Annu Rev Biochem 1987;56:289-315.
4. Andrews SC, Arosio P, Bottke W, Briat JF, von Darl M, Harrison PM, et al. Structure, function, and evolution of ferritins. J Inorg Biochem 1992;47(3-4):161-174.
5. Koorts AM, Viljoen M. Ferritin and ferritin isoforms I: Structure-function relationships, synthesis, degradation and secretion. Arch Physiol Biochem 2007;113(1):30-54.
6. Siimes MA, Addiego JE, Jr., Dallman PR. Ferritin in serum: diagnosis of iron deficiency and iron overload in infants and children. Blood 1974;43(4):581-590.
7. Shpyleva SI, Tryndyak VP, Kovalchuk O, Starlard-Davenport A, Chekhun VF, Beland FA, et al. Role of ferritin alterations in human breast cancer cells. Breast Cancer Res Treat 2011;126(1):63-71.
8. Alkhateeb AA, Han B, Connor JR. Ferritin stimulates breast cancer cells through an iron-independent mechanism and is localized within tumor-associated macrophages. Breast Cancer Res Treat 2013;137(3):733-744.
9. Ćujić D, Stefanoska I, Golubović S. Serum ferritin in healthy women and breast cancer patients. J Med Biochem 2010;30(1):33-37.
10. Kuvibidila SR, Gauthier T, Rayford W. Serum ferritin levels and transferrin saturation in men with prostate cancer. J Natl Med Assoc 2004;96(5):641-649.
11. Chou SF, Chen CY. Monoclonal and polyclonal antibodies against human ferritin, a nonspecific tumor marker. Hybridoma 2001;20(1):59-62.
12. Uesaka K. Production and clinical study of monoclonal antibodies against liver ferritin. Gan No Rinsho 1986;32(9):987-991.
13. Stevens RG, Graubard BI, Micozzi MS, Neriishi K, Blumberg BS. Moderate elevation of body iron level and increased risk of cancer occurrence and death. Int J Cancer 1994;56(3):364-369.
14. Lee CY, Leung WY, Tung JK. Applications of a monoclonal antibody to human ferritin in various immunoassays. Biotechnol Appl Biochem 1987;9(1):31-38.
15. Rath CE, Finch CA. Sternal marrow hemosiderin; a method for the determination of available iron stores in man. J Lab Clin Med 1948;33(1):81-86.
16. Lundin P, Lundquist A, Lundvall O. Evaluation of fine-needle aspiration biopsy smears in the diagnosis of liver iron overload. Acta Med Scand 1969;186(5):369-373.
17. Behjati Ardakani R, Ghods R, Bayat AA, Jeddi Tehrani M. Extraction and purification of ferritin from liver tissue. JBUM 2005;7(1).
18. Hadavi R, Zarnani AH, Ahmadvand N, Mahmoudi AR, Bayat AA, Mahmoudian J, et al. Production of monoclonal antibody against human nestin. Avicenna J Med Biotechnol 2010;2(2):69-77.
19. Loirat MJ, Gourbil A, Frioux Y, Muller JY, Blanchard D. A murine monoclonal antibody directed against the Gerbich 3 blood group antigen. Vox Sang 1992;62(1):45-48.
20. Kazemi T, Tahmasebi F, Bayat AA, Mohajer N, Khoshnoodi J, Jeddi-Tehrani M, et al. Characterization of novel murine monoclonal antibodies directed against the extracellular domain of human HER2 tyrosine kinase receptor. Hybridoma (Larchmt) 2011;30(4):347-353.
21. Mahmoudian J, Jeddi-Tehrani M, Bayat AA, Mahmoudi AR, Vojgani Y, Tavangar B, et al. A monoclonal antibody against leptin. Hybridoma (Larchmt) 2012;31(5):372-377.
22. Hajighasemi F, Shokri F. Production and characterization of mouse monoclonal antibodies recognizing multiple subclasses of human IgG. Avicenna J Med Biotechnol 2010;2(1):37-45.
23. Beatty JD, Beatty BG, Vlahos WG. Measurement of monoclonal antibody affinity by non-competitive enzyme immunoassay. J Immunol Methods 1987;100(1-2):173-179.
24. Cazzola M, Bergamaschi G, Tonon L, Arbustini E, Grasso M, Vercesi E, et al. Hereditary hyperferritinemia-cataract syndrome: relationship between phenotypes and specific mutations in the iron-

responsive element of ferritin light-chain mRNA. Blood 1997;90(2):814-821.

25. Orino K, Eguchi K, Nakayama T, Yamamoto S, Watanabe K. Sequencing of cDNA clones that encode bovine ferritin H and L chains. Comp Biochem Physiol B Biochem Mol Biol 1997;118(3): 667-673.
26. Boyd D, Jain SK, Crampton J, Barrett KJ, Drysdale J. Isolation and characterization of a cDNA clone for human ferritin heavy chain. Proc Natl Acad Sci USA 1984;81(15):4751-4755.
27. Mel'nikova IaI, Lunev VE, Preigerzon VA, Luneva NM, Koshkin SA, Rodionov MA, et al. Monoclonal antibodies to human spleen ferritin. II. Locali zation of epitopes and quantitative parameters of antigen binding. Biokhimiia 1993;58(5):759-771.
28. Nozawa S, Tsukazaki K, Narisawa S. Production of monoclonal antibodies to ferritin and development of the enzyme immunoassay system. Nihon Sanka Fujinka Gakkai Zasshi 1985;37(12):2775-2783.