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Original Article

Expression of Recombinant Insulin-Like Growth Factor-Binding Protein-3 Receptor in Mammalian Cell Line and Prokaryotic (*Escherichia coli*) Expression Systems

Abstract

Background: Insulin-like growth factor binding protein-3 receptor (IGFBP-3R) (Transmembrane protein 219 [TMEM219]) binds explicitly to IGFBP-3 and exerts its apoptotic and autophagy signalling pathway. Constructing a Henrietta Lacks (HeLa) h6-TMEM219 cell characterize the therapeutic potent of TMEM219 that could interrupt the IGFBP-3/TMEM219 pathway, in cancer treatment and destructive cell illnesses such as diabetes and Alzheimer's. **Materials and Methods:** First, to develop stable overexpressed HeLa h6-TMEM219 cells, and *Escherichia coli* BL21 (DE3) with high IGFBP-3R expression, the purchased pcDNA3.1-h6-TMEM219 plasmid was transformed and integrated using CaCl2 and chemical transfection reagents, respectively. The pcDNA3.1-h6-TMEM219 transfection and protein expression was evaluated by the polymerase chain reaction (PCR), western blotting, and flow cytometry. Following the induction of h6-TMEM219 expression, a protein was purified using Ni-NTA chromatography and evaluated by the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). **Results:** The 606 base pairs sequence in PCR outcomes confirmed successful pcDNA3.1-h6-TMEM219 transformation in *E. coli* BL21 and integration into the HeLa genome. The analysis of protein samples from induced *E. coli* BL21 and purified protein demonstrate a band of approximately 22 kDa on SDS-PAGE. Moreover, besides western blot analysis, flow cytometry findings illustrate approximately 84% of transfected HeLa cells (HeLa h6-TMEM219) overexpressed h6-TMEM219 on their surface. **Conclusion:** We designed a new experiment in the h6-TMEM219 expression procedure in both eukaryotic and prokaryotic hosts. All of our results confirm appropriate transformation and transfection and importantly, approve h6-TMEM 219 membrane expression. Finally, the HeLa h6-TMEM219 cells and the newly purified h6-TMEM219 leverage new studies for molecular diagnostic studies and characterize the therapeutic agents against IGFBP-3/TMEM219 signalling pathway in devastating illnesses *in vitro* and *in vivo*.

Keywords: *Alzheimer Disease, autophagy, cancer, diabetes mellitus, human, insulin-like growth factor-binding protein-3, TMEM219 protein*

Nima Naseri, Mina Mirian[1], Mohammad Reza Mofid

Departments of Clinical Biochemistry and [1]Pharmaceutical Biotechnology, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran

Introduction

Transmembrane protein 219 (TMEM219), also called insulin-like growth factor-binding protein 3 receptor (IGFBP-3R), is a novel death receptor and autophagy activator[1] that has been identified as an IGFBP-3 specific binding partner.[2] The human IGFBP-3R displays a 240-amino acid polypeptide consists of three domains including, (a) the extracellular N-terminal (IGFBP-3-connected) domain, which contains three phosphorylation sites[3] and three potential N-glycosylation sites, (b) The putative single-span transmembrane domain contains a unique leucine zipper sequence, and (c) The short C-terminal cytoplasmic domain (Caspase-8-connected).[2] Specific interaction of TMEM219 by IGFBP-3 in IGF/IGF-IR-independent pathway is responsible for the IGFBP-3-dependent cellular signaling processes such as triggering apoptosis,[2,4] regulation of autophagy,[1] and IGFBP-3-induced suppression of nuclear factor-κB activity in cancer cells.[5] Because of these performances, the IGF/IGF-IR-independent actions of IGFBP-3 have been shown to are related to the pathophysiology of human ailments such as cancer,[6,7] Type 1 diabetes mellitus (T1DM), asthma,[8] ischemia,[9,10] and Alzheimer's disease.[11] The obtained data from the various examinations were gathered TMEM219 expression is

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Address for correspondence:
Dr. Mohammad Reza Mofid, Department of Clinical Biochemistry, School of Pharmacy and Pharmaceutical Sciences, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: mofid@pharm.mui.ac.ir

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frequently suppressed in different malignancies such as prostate,[12] gastric,[13] lung,[14] and pancreatic cancer that associated with tumor progression and metastasis[15,16] The down-regulated TMEM-219 expression mitigates the IGFBP-3 apoptotic (inhibition) effect on cancer progression.[2] Subsequently, this receptor has been accepted as a novel anti-cancer death receptor that may naturally serve as a new diagnostic and prognostic biomarkers in specific cancers.[3] Unlike TMEM219 beneficial effects on cancer treatment, TMEM219 has an extensive destructive impact on the pathogenesis of T1DM[17,18] Hence, with respect to the remarkable role of the IGFBP-3/TMEM219 pathway in the pathophysiology of illnesses, and relying on comprehensive investigations, targeting a newly generated TMEM219 recombinant protein on cancer cells, may provide the conditions to the appraisal of TMEM219 agonists antibodies (mAbs) that may address as novel attractive diagnostic and the prognosis biomarker as well as the potential therapeutic of TMEM219[3] in treatment. In this respect, there is only one report regarding the production of the recombinant ecto-TMEM219.[17] Francesca D'Addio *et al*. expressed ecto-TMEM219 that comprised 161 amino acids of the extracellular N-terminal domain of intact TMEM219, in *E. Coli* systems as a reasonable host in protein expression. Whereas in the mentioned study, the protein expression and purification procedure have yet to be elucidated. Therefore, in the present study, we mean to devise a new procedure of expressing and purifying intact human protein 6 histidine-TMEM219, consist of all TMEM219 three domains, from both prokaryotic and eukaryotic cells category. The most identified recombinant protein expression platforms include prokaryotes, yeasts, and mammalian cell systems.[19] Mammalian cell hosts are more likely to produce appropriately, folded mammalian proteins with native-like post-translational modifications.[20] As mentioned above, TMEM219 has three N-glycosylation sites and three phosphorylation sites on its N-terminal domain. Therefore, according to the reasons stated to maintain the proper structure, folding and efficient performance of the human h6-TMEM219 protein, we expressed this protein in human Henrietta Lacks (HeLa) cells. Nevertheless, in a previous study, despite the expression of ecto-TMEM219 in *E. coli*, in the absence of post-translational modification, ecto-TMEM219 maintained its IGFBP-3 binding function. Therefore, constructing an h6-TMEM219 overexpressing *E. coli* BL21 (DE3*)* with the effect of isopropylthio-ß-D galactoside (IPTG) (Sigma-Aldrich) is reasonable and allows access to large amounts of h6-TMEM219, with maintaining nature function. The purified protein could be used as a soluble receptor protein in culture media and laboratory animals in future studies. The previous study has demonstrated that the expression of the soluble form of the N-terminal domain of the TMEM219 causes the receptor to bind to IGFBP-3, normalized circulating IGF-1/IGFBP3 levels, and hindered IGFBP3 deleterious effects *in vitro* and *in vivo*.[17] Finally, developing overexpressed HeLa h6-TMEM219 cells could serve the target protein needed to produce identifying probes in molecular diagnostic studies and characterize the therapeutic potent of TMEM219 agonists and antagonists that could interrupt the IGFBP3-TMEM219 pathway in cancer and destructive cell illnesses such as diabetes and Alzheimer.

Materials and Methods

Plasmid transformation and protein expression

The sequence of pcDNA3.1/Hygro (+) containing cloned h6-TMEM219 gene (NCBI accession number: Q86XT9) was designed and ordered to the General Biosystems Inc North Carolina, USA. The pcDNA3.1-h6-TMEM219 includes *Hygromycin-B-phosphotransferase* (Hygromycin resistance gene) as a known selectable marker of stable mammalian transfectants and *β-lactamase* (the Ampicillin resistance gene) as the selectable marker in the properly transformed prokaryotic hosts. The purchased pcDNA3.1-h6-TMEM219 was transformed into TOP10 *Escherichia coli* bacteria (Novagen, Madison, WI, USA) competent as a general bacterial plasmid amplification host and *E. coli* BL21 (DE3) competent cells as a general recombinant proteins expression host, cultured in the Luria–Bertani (LB) broth medium. At the first step of plasmid transformation, the permeability of the cell membrane was increased using calcium chloride (0.1 M). Then, 10 μL of plasmid were mixed and competent cells were simply transformed using the heat shock transformation method. Afterward, by adding 1 mL of ampicillin-free LB medium, the mixture was incubated and shaken for 1 h at 180 rpm. The transformed cells were first grown on LB agar (1.5%) medium supplemented with 100 μg ml^{-1} ampicillin (Roche, Germany) for 16 h at 37°C. Following amplification, several single fresh colonies of TOP10 *E. coli* were inoculated into a 5 ml LB medium supplemented with 100 μg ml^{-1} ampicillin. Furthermore, the recombinant *E. coli* BL21 (DE3*)* transferred to Terrific Broth media supplemented with 100 μg ml^{-1} ampicillin, 5% (v/v) glycerol, 12 g/L tryptone (Sigma-Aldrich, USA), 24 g/L yeast extract (Sigma-Aldrich, USA), 23.1 g/mL KH2PO4 and 125.4 g/mL K2HPO4, and incubate for 18 h. The cultured *E. coli* BL21 (DE3) cells were centrifuged at 5000 g and 25°C for 5 min to recover the bacterial pellet. Then, the cultures were incubated under 200 rpm of the shaking condition at 37°C for 2 h to an optimal density of OD_{600} 0.4–0.6, then the temperature was dropped to 25°C and over-expression of recombinant protein was induced by the addition of 1 mM IPTG. Afterward, the culture was incubated overnight. Finally, the transformed cells were centrifuged (5000 rpm, 15 min, 4°C), and the pellets were weighed and stored at −80°C until protein purification. Then, the plasmids were extracted from obtained pellets by GenElute™ Plasmid Miniprep

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Kit (Sigma-Aldrich) and digested by the *BglII* restriction enzyme (Thermo Fisher Scientific, USA), under the manufacturer's instruction.

Polymerase chain reaction

To approve the pcDNA3.1-h6-TMEM219 transformation in the *E. coli* BL21 (DE3) and transfection into the HeLa cells genome, a polymerase chain reaction (PCR) was accomplished on recombinant extracted plasmid cells using a pair of specific primers as described here: Forward primer: CTTCCTCCTCACCCACAG; Reverse primer: CCGGGTTCTAGACCAGTG. The primers identify a specific region of the h6-TMEM219 sequence on the plasmid and genomic DNA. The PCR manner was commenced by one cycle at 95°C for 5 min, proceed with 30 cycles at 95°C for 15 s, 65°C for 15 s, 72°C for 45 s, and finished with one cycle at 72°C for 5 min at a thermocycler (Bio-Rad, USA).

Escherichia coli BL21 (DE3) cell lysis and protein extraction by Ni-NTA column

First, 300 mg of *E. coli* BL21 (DE3) cell pellets was resuspended in 5 mL lysis buffer containing 25 mM Tris-HCl (pH: 8), 250 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 µg/mL *DNAse*/*RNAase* (Thermo scientific, USA) and homogenized for 5 min to purify h6-TMEM 219 as a soluble protein. Then, to overstate the efficiency of protein extraction, 1 mg/mL lysozyme (SolarBio, China) and 2 mg/mL N-decyl-B-D maltopyranoside (Atgrade, USA) was added to the mixture and incubated at room temperature for 2 h N-decyl-B-D maltopyranoside is a nonionic detergent used to purify integral membrane proteins.[21] Then, the lysate was centrifuged at 5000 rpm and 4°C for 15 min. Afterward, the supernatant was run to the affinity Ni-NTA column to purify 6 histidine-TMEM219. Then, the Ni-NTA was washed by A buffer (25 mM Tris-HCl, 50 mM NaCl, pH: 8), and competing B buffer (25 mM Tris, 0.5 M NaCl, and 1 M imidazole) to segregate the h6-TMEM219 from the Ni-NTA column.

Protein separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis

The protein samples from transformed and untransformed *E. coli* BL21 (DE3) and protein solutions extracted from the column were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) electrophoresis. Then, all samples were mixed with 5X loading buffer (0.5% (w/v) bromophenol blue, 0.5 M Tris-HCl pH: 6.8, 20% (v/v) glycerol and 2% SDS), 5% (v/v) β-mercaptoethanol (Sigma-Aldrich, USA) and boiled for 5 min at 96°C. Then, 20 µL of each sample and 8 µl of low-molecular weight and high-molecular weight protein ladder (Pharmacia Biotech, USA) were loaded onto 15% polyacrylamide gel (GE Healthcare, USA) and run for 40 min at 1X Tris solution running buffer (25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS, pH: 8.3) (BIO-RAD, USA) at 15 mA. Finally, the gels were stained with coomassie blue.

Cell culture and transfection

The HeLa cells were purchased from the Pasteur Institute (Tehran, Iran) and were cultured in Dulbecco's Modified Eagle Medium (Sigma, USA) supplemented with 10% FBS (Bioidea, Iran), 100 µg streptomycin (Gibco, USA) at 37°C and 5% CO_2. Hela cells transfection was conveyed applying isolated pcDNA3.1/Hygro (+)-h6-TMEM219 and TurboFect transfection reagent (Thermo Fisher Scientific, USA) to establish overexpressed transfected HeLa cells (HeLa h6-TMEM219). In brief, at the logarithmic growth stage, 4×10^5 cells/well was seeded in 6 well plates to reach 70%–80% confluency. Transfection was carried out using 400 microliters (10 ng/µL) of the linear plasmid and 8 µl of TurboFect transfection reagent in serum-free media. Then, after 1 day, the medium was changed, and the cells incubated for 48 h. Then, the media was replaced with fresh medium-containing different doses of Hygromycin B (Invitrogen, USA) to expand the HeLa h6-TMEM219 resistant cells. For 3 weeks, the cells were treated with Hygromycin, ongoingly enhanced from 100 µg ml^{-1} in the 1st week to 200 µg ml^{-1} in the 2nd week and t to 400 µg ml^{-1} in the 3rd week for the cell's screen with different copy numbers of the inserted gene. The HeLa h6-TMEM219 was further subjected to 200 µg ml^{-1} hygromycin selection during the study to obtain stable transfectants.

Flow cytometry

The h6-TMEM219 protein appearance on the HeLa h6-TMEM219 cell membrane was evaluated based on PE anti-His tag mAb (BioLegend, USA) interaction with 6 his-TMEM219. The cells were initially harvested by a sterilized Trypsin-EDTA (0.25%) (Gibco™) and washed two times with Phosphate-buffered saline (PBS) containing 1% FBS. Approximately 3×10^5 of HeLa h6-TMEM219 and controlled cells were incubated with the 2 µl PE anti-His tag mAb in 500 µl PBS, for 30 min at 4°C. Then, the cells were centrifuged at 1200 rpm for 10 min and washed with PBS/containing 1% FBS. Finally, the cells were re-suspended in 200 µl PBS, and the fluorescence signals of Phycoerythrin (PE) intensity were detected using FACS Calibur (BD Biosciences, USA). The gathered information was visualized by FlowJo software (Tree Star, USA).

Western blot analysis

Protein expression in the HeLa h6-TMEM219 cells was confirmed by Western immunoblot assay using the anti-His tag mAb (BioLegend). The cell protein component isolated from both HeLa h6-TMEM219 and untransfected cells using radioimmunoprecipitation assay buffer (Thermo Scientific, USA) and (protease and phosphatase) inhibitor (1 mM PMSF, DTT 1 mM, Leupeptin 10 µg/ml, Pepstatin A 1 µg/ml, Aprotinin 60 µg/ml). Then, equal amounts

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of samples (50 µg) in e conditioned media were boiled for 10 min and separated by 10% SDS-PAGE gel. Then to protein detection by anti-His tag mAb antibody, the proteins were transferred (400 mA, 1 h) on to a polyvinylidene fluoride membrane and nonspecific binding was blocked by placing the membrane in 2.5% nonfat milk (5% bovine serum albumin, 0.2% Tween 20 and Tris-buffered saline) for 1 h. Subsequently, primary anti-His tag mAb (1:1000 dilution in 2.5% nonfat milk) was added and incubated at the room temperature for 2 h. After three washes with 2.5% nonfat milk (each for 10 min), a secondary anti-mouse antibody conjugated to horseradish peroxidase (conjugated secondary Ab) (Sigma, USA) was added and shook at the room temperature for 2 h. Then the membrane was washed three times in 2.5% nonfat milk, and the bound secondary antibodies were measured with the ECL Western blotting detection system (Takara Inc., Kyoto, Japan).

Results

Amplification of pcDNA3.1-h6-transmembrane protein 219 and construct confirmation

In addition to Sanger sequencing of the purchase plasmid, performed by General Biosystems Inc (Morrisville, NC, USA), the accuracy of extracted pcDNA3.1/amp (+)-h6-TMEM219 from *E. coli* TOP 10 was assess using DNA electrophoresis for purchased and linearized plasmid. The observation of 6257 bp linearized pcDNA3.1/Hygro (+)-h6-TMEM219 in agarose gel electrophoresis proved the correct plasmid generation, transformation, and plasmid linearization [Figure 1a]. Furthermore, the PCR reaction on HeLa h6-TMEM219 cells genomic DNA, and the plasmid purified prokaryotic cells with a pair of TMEM219 backbone primers, represent a single sharp band 606 bp, verifying plasmid transformation and insertion in *E. coli* and HeLa genome, respectively, as depicted in Figure 1b.

Expression and induction of h6-transmembrane protein 219

The theoretical size of h6-TMEM219 protein was calculated based on the ExPASy proteomics Web server (http://www.expasy.org), considering 38 amino acid signal peptides cleavage, and the proteins' Rf as compared to the molecular weight of the protein ladder standards as 22 kDa. By comparing induced *E. coli* BL21 (DE3) SDS-PAGE pattern with untransformed bacteria, the expression of 22 kDa h6-TMEM219 in induced *E. coli* BL21 (DE3) confirmed the successful pcDNA3.1/amp (+)-h6-TMEM219 transformation and expression in *E. coli* BL21 (DE3) [Figure 2a]. Since the h6-TMEM219 promoter was under the control of the lac operon in pcDNA3.1, protein expression is considerably dependent on IPTG. As shown in Figure 2b, h6-TMEM219 expression arises at the 6 and 12 h after 1 mM IPTG induction.

Protein extraction by Ni-NTA column

The SDS-PAGE results of the first stage of h6-TMEM219 purification elucidate that a considerable part of h6-TMEM 219 protein remained in the sediment; therefore, NaCl solution was not able to h6-TMEM219 as a soluble protein [Figure 3a]. As can be seen, after Ni-NTA column washing with buffer A, most of the h6-TMEM219 (22 kDa) was removed (fourth column). Whereas following, washing with imidazole buffer (1 M), an insufficient amount of h6-TMEM219 was purified.

Protein purification h6-transmembrane protein 219 with N-decyl-BD maltopyranoside

Due to insufficient protein purification in the previous step, at the second step to enhanced the protein extraction efficiency, we added 2 mg/mL N-decyl-B-D maltopyranoside (DDM) lysozyme buffer characterize for integral protein purification. The SDS-PAGE results demonstrate that unlike the previous step of purification in the second step in the presence of DDM, following Ni-NTA column washing with the competing imidazole buffer, as increasing of imidazole concentration (fourth and seventh columns), the large amount of h6-TMEM219 with 22 kDa was extracted from Ni-NTA column [Figure 3b].

Flow cytometry analysis

Using flow cytometry, PhycoErythrin (PE)-fluorescence intensity ratio illustrates that remarkably, 84% of HeLa

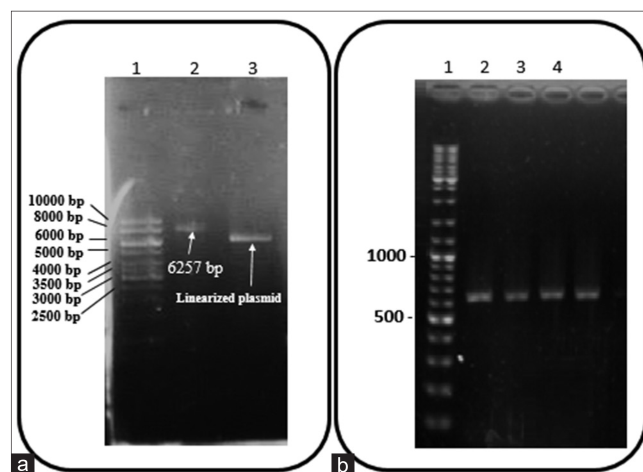

Figure 1: (a) The restriction enzyme digestion was performed using BglII to verify the accuracy of purchased pcDNA3.1/Hygro (+) h6 Transmembrane protein 219 and to linearized plasmid for transfection assay. Lane 1: DNA ladder 1Kb; lane 2: Extracted circular plasmid; lane 3: Single digestion with BglII. (b) Polymerase chain reaction analysis on genomic DNA of transfected Henrietta Lacks cells and purified pcDNA with pcDNA backbone primers. Lane 1) Mix DNA ladder 1 kb (Thermo Scientific, USA). (Lane 2) Henrietta Lacks h6 TMEM219 cells genome, 606 bp fragment corresponding to the transfected plasmid. (Lane 3) Extracted pcDNA3.1 h6 Transmembrane protein 219 from transformed Escherichia coli TOP10 cells. (Lane 4) Extracted pcDNA3.1 h6 Transmembrane protein 219 from transformed Escherichia coli BL21 (DE3) cells

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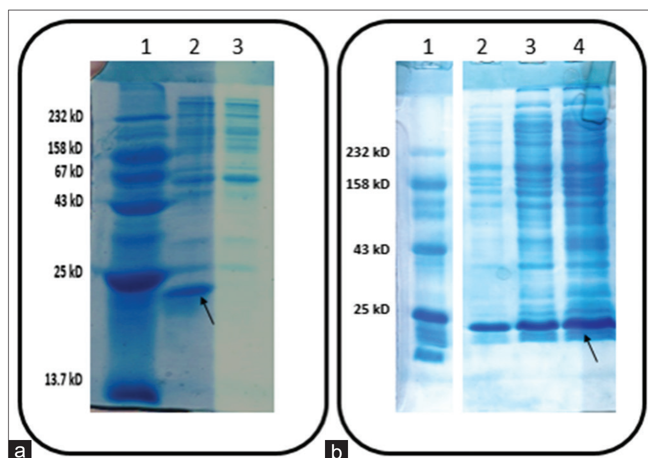

Figure 2: (a) Fifteen percent sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of h6-Transmembrane protein 219 expressed in *Escherichia coli* BL21 (DE3). (1) Low-molecular-weight and high-molecular-weight protein ladder. (2) The induced bacteria containing recombinant pcDNA3.1-h6-Transmembrane protein 219. (3) The untransformed bacteria without pcDNA3.1-h6-Transmembrane protein 219. (b) Fifteen percent sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the induction process h6-Transmembrane protein 219 protein. (1) Low molecular weight and high molecular weight protein ladder (2) The un-induced *Escherichia coli* BL21 (DE3) containing recombinant pcDNA3.1-h6-Transmembrane protein 219. (3) The induced *E. coli* BL21 (DE3) containing recombinant of pcDNA3.1-h6-Transmembrane protein 219 h6-Transmembrane protein 219 expressions after IPTG addition. (4) The induced *E. coli* BL21 (DE3) containing recombinant of pcDNA3.1-h6-Transmembrane protein 219 h6-Transmembrane protein 219 expressions after 6 h of induction with IPTG. (4) The induced *Escherichia coli* BL21 (DE3) containing recombinant of pcDNA3.1-h6-Transmembrane protein 219 after 12 h of induction with isopropylthio-ß-D galactoside

h6-TMEM219 cells overexpressed h6-TMEM219 on their surface compared with negative control cells ($P < 0.001$) [Figure 4].

Western blotting

The Western blot analysis using His-tag specific mAb (BioLegend) confirmed the IGFBP-3R (TMEM219) protein expression on the HeLa h6-TMEM219 cells, revealed that the target protein of 22 kD was expressed [Figure 5].

Discussion

TMEM219, known as IGFBP-3 specific binding partner, is located in the plasma membrane and the perinuclear and cytoplasmic areas.[2,22] The TMEM219 plays a vital role in IGFBP-3 anti-proliferative[2] and autophagy effect on tumor suppression.[1,2,5,14,23] Nevertheless, besides IGFBP-3/TMEM219 hindrance impact on cancer progression, this pathway has a wrecking effect on the deterioration of destructive cell illnesses such as diabetes, asthma,[8] and disruption of colonic stem cell function in a preclinical diabetic enteropathy patient.[17] Furthermore, in IGFBP-3 independent pathway, TMEM219 mediates diverse signaling and effector responses of chitinase 3-like-1[24,25] The crucial role of TMEM219 in the pathophysiology of various illnesses persuaded us to

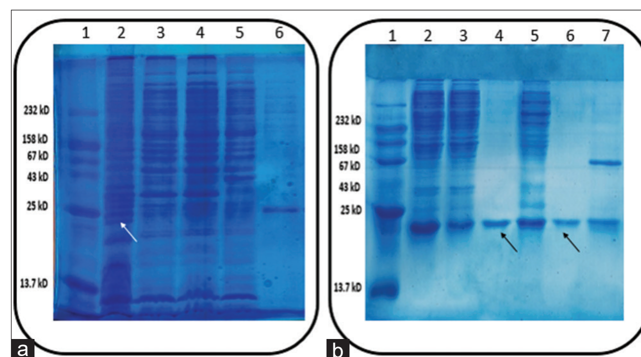

Figure 3: (a) 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of the first stage of purification process h6-Transmembrane protein 219 protein with the Ni-NTA column. (1) low molecular weight and high molecular weight protein ladder. (2) The protein sample of cell pellets. (3) The protein sample before injecting to the column. (4) The protein sample after injecting to the column. (5) The protein sample after washing elution buffer A. (6) The protein sample after washing 0.5M of imidazole buffer. (b) Fifteen percent sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of h6- Transmembrane protein 219 protein purification process using N-decyl-B-D maltopyranoside and the Ni-NTA column. (1) low molecular weight and high molecular weight protein ladder (2) The protein sample before injecting to the column. (3) The protein sample after injecting to the column. (4) The protein sample after 0.5M of imidazole buffer. (5) The protein sample after washing buffer A. (6) The protein sample after 1M of imidazole buffer. (7) The protein sample after 1M of imidazole for the second time

perform new research about TMEM219 protein expression and purification might be having promising results for developing potential therapeutic compounds like TMEM219 agonist[3] and antagonists for human diseases treatment in future studies. In this respect, Francesca D'Addio and *et al*. have reported the expression of the extracellular domain of TMEM219 (ecto-TMEM219) in *E. Coli*,[17] in contrast, the results about the transformation, protein expression procedure, ecto-TMEM219 amino acid sequence, and protein purification protocol have yet to be elucidated. The findings from previous studies demonstrate that posttranslational modifications affect protein properties relevant to their folding[26] and therapeutic application.[27] Thus, in Francesca D'Addio *et al*. study,[17] ecto-TMEM219 expression in the absence of transmembrane and C-terminal domain along with the absence of post-translational modifications in *E. coli* makes this presumption that ecto-TMEM219 may not have the real and same function as nature human TMEM219. Therefore, contrary to previous assumptions, we assume that ecto-TMEM219 expression in *E. coli* has no scientific reason for developing therapeutic agents like monoclonal antibodies. As compared to our study, the main differences between the Francesca D'Addio and *et al*. study and ours are: (1) The expression of intact h6-TMEM 219 containing TMEM219 N-terminal, transmembrane, and C-terminal domain, (2) h6-TMEM219 expression in the cancerous eukaryotic cells and favoring the soluble secretion of the h6-TMEM219 recombinant protein in the prokaryotic category,[3] Approving h6-TMEM219 membrane expression by anti His tag in flow cytometry, 4) Setting-up

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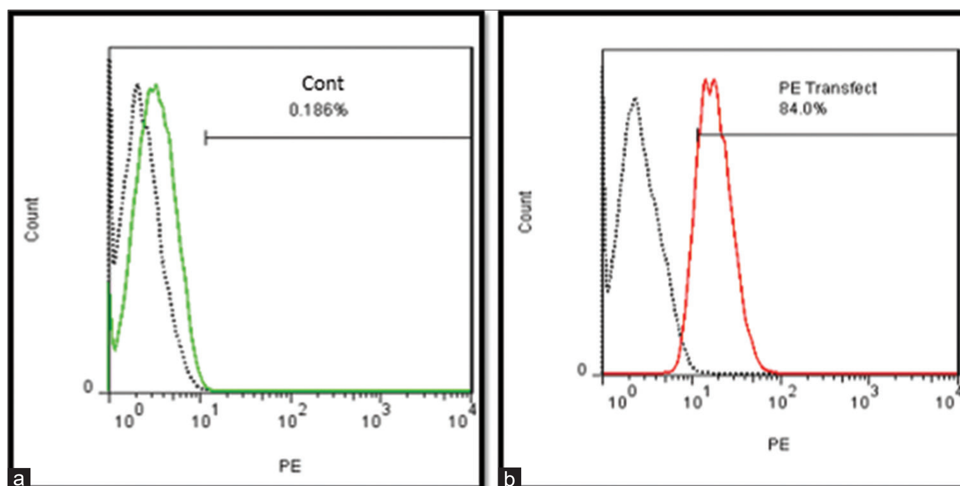

Figure 4: Flow cytometry analysis of h6 Transmembrane protein 219 membrane expression using PE anti His tag mAb against h6 Transmembrane protein 219. (a) Untransfected Henrietta Lacks cells and control isotype. (b) Henrietta Lacks h6 TMEM219 cells and control isotype

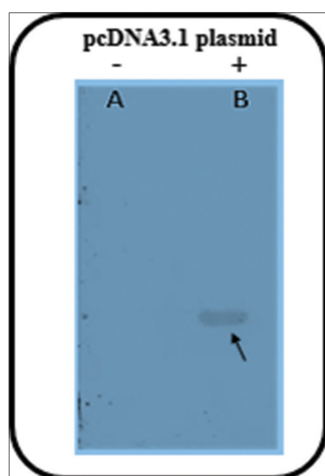

Figure 5: Western blot analysis of overexpressed Henrietta Lacks containing recombinant pcDNA3.1 h6 Transmembrane protein 219 plasmid using an anti His antibody. (a) Extract protein from Henrietta Lacks cells containing pcDNA3.1 h6 Transmembrane protein 219. (b) Extract protein from Henrietta Lacks cells without pcDNA3.1 h6 Transmembrane protein 219

the TMEM219 purification manner by DDM lysis buffer and NTA‑column. Posttranslational modification of the h6‑TMEM219 and its intact expression in eukaryotic HeLa cells causes to maintain h6‑TMEM219 proper structure, folding, and function. In the current examination, we performed PCR assay, western blotting, and flow cytometry as standard approving tests in protein expression to evaluate the h6‑TMEM219 expression process in *E. Coli* and HeLa cells. At the molecular level, the observation of the 606 bp band in the PCR technique approves the proper transformation of pcDNA3.1‑h6‑TMEM219 in *E. coli* cells and its integration in the HeLa cells genome. The pcDNA3.1 produced h6‑TMEM219 recombinant protein would precipitate as membrane receptor. Therefore, in the current investigation, h6‑TMEM219 expression in HeLa cells was confirmed using western blotting and flow cytometry. The obtained outcomes from the flow cytometry assay represented that approximately 84% of HeLa h6‑TMEM219 cells $P < 0.001$, expressed h6‑TMEM219 on their cell surface is compared with negative control cells.

Following a previous study[2] and as can be seen in our identified result, TMEM219 expressed as a membrane protein could have different purification strategies from other cell proteins.[28] In the current study, due to the low probability of h6‑TMEM219 expression as a membrane protein in bacteria, first, we try to purify h6‑TMEM219 as a soluble protein from cell sediment by using 1 mg/ml lysozyme and 250 mM NaCl. h6‑TMEM219 retention in cell sediments illustrates that h6‑TMEM219 probably not expressed as a peripheral protein. Interestingly, following using DDM used purifying the integral membrane receptor proteins,[29] the large amount of h6‑TMEM219 was purified, which strengthens our hypothesis, that h6‑TMEM219 might be expressed as a membrane protein in *E. coli* BL21 (DE3). Finally, according to suppressed expression of TMEM219 in cancer cells[2,5,14,23] constructing the h6‑TMEM219 overexpressed HeLa h6‑TMEM219 cells as a bonafide IGFBP‑3 receptor may serve as new diagnostic and prognostic biomarkers in cancers and allow more efficient characterizing of TMEM219‑coupled therapeutics in cancer treatment.[3] The h6‑TMEM219 expression in prokaryotic cells allows evaluating the binding potency of h6‑TMEM219 to interact with IGFBP‑3 in future studies. Regardless of the status of TMEM219 expression, in the future study, we intend to assess the possible protective and therapeutic effect of newly generated h6‑TMEM219 on chi3 responses and abrogating IGFBP‑3 deleterious effects by block the downstream signaling of IGFBP‑3/TMEM219 pathway in devastating destructive cell disease such as diabetes and Alzheimer's that may constitute to paves the way for a novel potential therapeutic target *in vitro* and *in vivo*, which requires further studies in this area.

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Conclusion

We devised and optimized a new manner to express intact h6-TMEM219 in both eukaryotic (HeLa) and prokaryotic *(E. coli)* hosts, with the different procedures including transformation, IPTG induction, turbofect polymeric transfection, western blotting, and flow cytometry. All of our study results approved appropriate transformation and transfection and importantly, h6-TMEM 219 membrane expression. The overexpressed HeLa h6-TMEM219 cells and the newly purified h6-TMEM219 serve as the target protein leverage doing the new studies to produce identifying probes in molecular diagnostic studies and characterize the therapeutic compound against IGFBP-3/TMEM219 signaling pathway in different disorders *in vitro* and *in vivo* and assess its diagnostic value and its potential as a therapeutic target.

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Conflicts of interest

There are no conflicts of interest.

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