Gene 591 (2016) 433–441

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Snail represses the expression of human phospholipid scramblase 4 gene

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ARTICLE INFO

Article history:
Received 29 February 2016
Received in revised form 9 June 2016
Accepted 24 June 2016
Available online 27 June 2016

Keywords:
Promoter activity
Transcriptional regulation
Phospholipids
Chromatin immunoprecipitation assay

ABSTRACT

Human phospholipid scramblases (*hPLSCRs*) are a group of transmembrane ATP independent lipid transporters mediating bi-directional transport of phospholipids. There are four homologues *hPLSCR1-hPLSCR4* and *hPLSCR1* is the extensively studied homologue among them. *hPLSCR4* shares 48% homology with *hPLSCR1* and mediates scrambling of PLs similar to *hPLSCR1* in Ca^{2+} dependent manner. Transcriptional regulation helps in better understanding of the function and the expression of a protein. Till date there are no reports suggesting the transcriptional regulation of *hPLSCR4*. In this study, we identified Snail to be a potent regulator of *hPLSCR4*. ConSite tool predicted the presence of a putative Snail binding site with a consensus sequence of $^{-1521}$CAGGTG^{-1516} on *hPLSCR4* promoter. Luciferase assays depicted a dose dependent decrease in *hPLSCR4* promoter activity with an increase in amount of Snail. Deletion analysis revealed that the region from −1380 to −2100 to be the regulatory region of hPLSCR4. Knock down studies further confirmed Snail mediated downregulation of hPLSCR4, as the mRNA and the protein levels of *hPLSCR4* considerably increased under knock down conditions. The *in vivo* interaction of Snail with *hPLSCR4* promoter was further confirmed by ChIP assay. This is the first report on the transcriptional regulation of *hPLSCR4*, where Snail was shown to downregulate the expression of *hPLSCR4*.

1. Introduction

Human phospholipid scramblases (*hPLSCRs*) are a group of homologues, ATP independent, Ca^{2+} dependent lipid transporters which mediate bi-directional transport of phospholipids (PLs) across the phospholipid bi-layer (Sahu et al., 2007). Four homologues of scramblases have been identified till date, which include *hPLSCR1-hPLSCR4*. Among all the isoforms, *hPLSCR1* has been extensively studied and is a multifunctional protein with roles in apoptosis (Bailey et al., 2005), antiviral defense (Dong et al., 2004), cell differentiation and proliferation (Zhou et al., 2002). *hPLSCR1* is also known to play active roles in a variety of cancers like metastatic liver cancer (Cui et al., 2012), colorectal cancer (Kuo et al., 2011) and ovarian cancer (Silverman et al., 2002). *hPLSCR4* homologue is not well characterized yet, even though blast searches revealed 48% sequence homology of *hPLSCR4* with *hPLSCR1*. *hPLSCR4* encodes for a protein of 329 aa and is generally localized to PM (Wiedmer et al., 2000). Multiple sequence alignment results revealed similarity between Ca^{2+} binding domains of *hPLSCR1* and *hPLSCR4* and showed similar Ca^{2+} binding properties. Mutation of residues Asp^{290}-Ala in the Ca^{2+} binding motif of *hPLSCR4* resulted a reduction in scrambling activity. To characterize the functional importance of *hPLSCR4*, recombinant protein was expressed, purified and reconstituted into artificial bi-layers. *hPLSCR4* was shown to mediate bi-directional transport of PLs across the bilayer which was metal ion dependent (Francis and Gummadi, 2012). *hPLSCR1* has additional roles in the nucleus as it enters nucleus *via* NLS and binds with IP3R1 receptor and regulates its expression (Zhou et al., 2005). Reports suggest that NLS of *hPLSCR4* binds to minor localization signal of importin α which also helps in trafficking it to the nucleus (Lott et al., 2011) while other functions of *hPLSCR4* apart from scrambling are not yet elucidated. Based on sequence similarity and the presence of conserved domains, *hPLSCR4* might also have many functions like *hPLSCR1*. Transcription factors (TF) play an important role in regulating the expression of any gene. Transcriptional regulation might help in better understanding of the protein, its expression levels and functions. Till date there are no reports suggesting transcriptional regulation of *hPLSCR4*, and there are two reports on transcriptional regulation of *hPLSCR1*. Snail was shown to downregulate the expression of *hPLSCR1* in IMR-32cells (Francis et al., 2014) and c-Myc was shown to up regulate the expression of *hPLSCR1* in HEK 293 cells (Vinnakota and Gummadi, 2016). Online

Abbreviations: hPLSCR4, human phospholipid scramblase 4; β-Gal, betagalactosidase; TF, transcription factor; PL, phospholipids; RLU/β-gal, relative luciferase assay/beta galactosidase assay; PM, plasma membrane; PS, phosphatidyl serine; shRNA, small hairpin RNA.

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http://dx.doi.org/10.1016/j.gene.2016.06.050
0378-1119/

prediction tools suggested a putative Snail binding site on *hPLSCR4* promoter. We hypothesized that Snail might transcriptionally regulate the expression of *hPLSCR4*. To prove this hypothesis, reporter gene assays, ChIP and knock down studies were carried out and our results clearly indicated the transcriptional regulation of hPLCR4 by Snail.

2. Materials and methods

2.1. Cell culture

HEK 293, Huh-7 and U-87 MG cell lines were maintained in DMEM (Hyclone, Logan UT, USA) medium supplemented with 10% FBS (Thermo Fisher Scientific, USA) along with antibiotics and Antimycotic (Thermo Fisher Scientific, USA) at 37 °C in a 5% CO_2 incubator.

2.2. Bioinformatic analysis

The promoter of *hPLSCR4* (~2.1 kb) was screened for putative transcription factor binding sites using a TF prediction tool called ConSite (http://consite.genereg.net/). The search for putative TF binding sites was performed at 100% cutoff. The results were then compared with other TF prediction tools like Alibaba, TF Search and Tfsitescan.

2.3. Cloning the hPLSCR4 promoter reporter construct

The 5′ flanking region of *hPLSCR4* promoter ranging from −2017 bp to +105 bp was selected and PCR amplified from human genomic DNA template using Phusion high fidelity polymerase (New England Biolabs, USA). The primers used for amplifying the 2122 bp region of *hPLSCR4* promoter include *hPLSCR4* forward primer 5′-AATTAGGTACCGTCATCC CTCGAAATGTCCAGGTTG-3′ and *hPLSCR4* reverse primer 5′-CCCTCG AGCGGAGAGGATTTTTCAAGTTAT - 3′. The following conditions were used for amplifying the promoter: 95 °C — 5 min, 98 °C — 30 s, 67 °C — 40 min, 72 °C — 1 min and 72 °C — 10 min. The amplified fragments were run on a 1% agarose gel and visualized under UV transilluminator. The amplified 2.1 kb fragment was specifically cloned between *Kpn*I and *Xho*I sites of a pGL3 basic vector. The clone was further confirmed by sequencing.

2.4. Cloning the expression plasmids

Snail expression plasmid was generated by cloning the cDNA of Snail transcription factor into pcDNA3.1 between *BamHI* and *XhoI* sites. c-Myc expression plasmid was generated by cloning the cDNA of c-Myc transcription factor into pcDNA3.1 between *BamHI* and *EcoRI* sites.

2.5. Transfections and luciferase assays

HEK 293 cells (60–70% confluent) were transfected with *hPLSCR4* promoter reporter construct, Snail expression plasmid, β-galactosidase plasmid using calcium phosphate method (Kingston et al., 2003) in 24 well plates. After 24 h of transfection, cells were lysed in cell lysis buffer [100 mM phosphate buffer (pH 7.8), 1 mM DTT, 0.1% (w/v) Triton-×100] and luciferase activity was assayed in assay buffer [100 mM Tris acetate (pH 7.8), 10 mM magnesium acetate, 1 mM EDTA, 2 mM ATP (Bio Basic, Canada) and 1 mM D-luciferin (Sigma-Aldrich, USA)]. The relative luminescence readings were measured in a luminometer (Berthold Detection Systems, Germany). Cells were simultaneously assayed for β-galactosidase activity by measuring the absorbance at 462 nm after incubating with the substrate ONPG (Sigma-Aldrich, USA) at 37 °C for 10–15 min. Similarly, the effect of the TF c-Myc on *hPLSCR4* was also studied by transfecting HEK 293 cells with c-Myc expression plasmid and hPLSCR4 promoter reporter constructs using calcium phosphate method of transfection (Kingston et al., 2003). Luciferase assays were performed as per the method described earlier.

2.6. Western blot

HEK 293 cells were transfected with Snail expression plasmid and pcDNA3.1 as control plasmid. 24 h post transfection, cells were lysed in RIPA buffer [150 mM NaCl, 1 mM EDTA, 50 mM Tris-HCl (pH 7.4), 1% (v/v) NP-40, 0.5% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 1 mM PMSF and protease inhibitor cocktail (Sigma-Aldrich, USA)] and the total protein was estimated using BCA kit (Sigma-Aldrich, USA). 30–50 μg of total cell lysate was loaded on 12% SDS-PAGE and then transferred onto a nitrocellulose membrane (Pall lifesciences, USA). The nitrocellulose membrane was then blocked for 2 h at 25 °C in blocking buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.2% (v/v) Tween-20) with 5% (w/v) skimmed milk]. The blots were incubated using primary goat anti-*hPLSCR4* antibody (Santa Cruz, USA) and primary mouse anti-Snail antibody (Santa Cruz, USA) at 4 °C for 8 h. The resulting blot was washed thrice with TBST and probed with donkey anti-goat secondary antibody (Santacruz, USA) and goat anti-mouse secondary antibodies (Santacruz, USA) respectively. The blots were simultaneously probed for Actin using mouse anti-Actin primary antibody. The immunoblots were developed using chemiluminescence kit (Bio-rad, USA).

2.7. Deletion analysis and plasmid construction

Deletion constructs lacking the Snail binding region were constructed. Two deletion constructs were generated: −1380 deletion construct and −650 deletion construct. −1380 deletion construct was amplified employing a forward primer 5′-GG GGTACCGCTTGCTCCCAA CAGTAACATATG 3′ and −650 deletion construct was amplified employing a forward primer 5′-GGGGTACCATCTCACGAGGTCAGGAG ATC-3′. The reverse primer which was used to amplify the full length *hPLSCR4* promoter of size 2.1 kb was employed for the amplification of the deletion constructs. The deletion constructs were cloned into *Kpn*I and *Xho*I sites in the MCS of pGL3 basic vector.

2.8. Site directed mutagenesis

Point mutations were induced in Snail binding site on *hPLSCR4* promoter by site directed mutagenesis. The forward primer 5′-CCTGAGCG GGCATGGTGTTCCTCACCC-3′ and the reverse primer 5′-GGGTGAGGAA CACCATGCCCGCTCAGG-3′ carrying mutations in Snail binding site were employed. The reaction mixture of total volume 12.5 μl was set up using 50 ng of *hPLSCR4* promoter reporter construct as template, 1.25 μl of 5× Phusion high fidelity buffer, 2 μl of 10 μM mutated forward and reverse primers each, 1 μl of 10 mM dNTPs and 0.25 μl of 2 U/μl Phusion high fidelity polymerase and 5 μl of water. The PCR program employed for inducing point mutations in predicted Snail binding site is as follows: 98 °C — 5 min, 98 °C — 40 s, 62 °C — 50 s, and 72 °C — 15 min, for 15 cycles, followed by a final extension of 72 °C — 10 min. The PCR product was then checked for the presence of the band of desired size (2.1 kb) and digested with *Dpn*I (New England Biolabs, USA) for 12 h. The resulting digested product was transformed into DH5αcells and screened for colonies which are positive for the desired mutation. The clones were further confirmed by sequencing.

2.9. RNA isolation and RT PCR

HEK 293 cells (60–70% confluent) were transfected with Snail shRNA plasmid (Santa Cruz, USA) and control shRNA plasmid (Santa Cruz, USA). After 24 h of transfection, total RNA was isolated by TRIZOL reagent, and cDNA was synthesized using the cDNA synthesis kit (Takara Bio, USA) as per the manufacturer's instructions.