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Snail interacts with hPLSCR1 promoter and down regulates its expression in IMR-32

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ABSTRACT

Human phospholipid scramblase 1 (hPLSCR1) is a proapoptotic protein whose expression is deregulated in a variety of cancers cells. However till date the transcription regulation of hPLSCR1 is unknown. Transcriptional regulation of hPLSCR1 was studied by cloning the 5′-flanking region of hPLSCR1. Luciferase assays revealed that −1525 to −1244 region of hPLSCR1 was found to regulate its promoter activity. A putative Snail transcription factor (TF) binding site was found within the regulatory region of the promoter. Snail binding was found to down regulate the expression of hPLSCR1 both at the transcriptional and translational levels. Snail knock down using Snail-shRNA confirmed that down regulation of hPLSCR1 by Snail was specific. Point mutation studies confirm that the predicted Snail TF binds to −1123 to −1117 site. ChIP assay further confirms the physical interaction of Snail with hPLSCR1 promoter. This is the first report showing the transcriptional regulation of hPLSCR1 expression by Snail TF and its possible implications in cancer progression.

1. Introduction

Epithelial-mesenchymal transcription (EMT) is a crucial process required for the polarization of epithelial cells to invasive mesenchymal phenotype [1]. Important event during EMT transition involves down-regulation of cell adhesion molecule E-cadherin and till date several repressors of E-cadherin have been identified and termed as EMT regulators (EMTRs). EMTRs include Snail, Slug, ZEB1, SIP1 and Twist which are important for transition of epithelial cells to melanoma cells and also contribute to resistance towards apoptosis, senescence and immune system evasion [2,3]. Snail and Slug which belong to the E-box class of transcription factors are the most important transcription factors which regulate E-cadherin expression [4]. Initially Snail was implicated in the differentiation of epithelial cells to mesenchymal cells during embryo development; however the same molecule has been shown to be crucial for tumor progression. Snail transcription factor is an important zinc finger protein which effects EMT transition via down regulation of E-cadherin and up-regulation of vimentin [5]. Expression of Snail is up-regulated in several cancers such as colorectal cancer, renal cell carcinoma and myeloid cancer underlying the importance of this molecule in tumor invasion and metastasis.

hPLSCR1 is a type II integral membrane protein belonging to the ATP-independent class of phospholipid translocators which mediate bidirectional scrambling of phospholipids (PLs) in a Ca^{2+} dependent manner. It is a multi-domain protein which effects bidirectional scrambling of PLs particularly phosphatidylserine (PS) which acts as a signal for apoptosis, blood coagulation and cell activation events [6]. In hPLSCR1−/− null mice, blood coagulation was normal however defective proliferation and differentiation of hematopoietic cells was observed [7]. Hence hPLSCR1 is not considered as a true scramblase despite mediating transbilayer movement of PLs in synthetic vesicles [8]. hPLSCR1 is known to shuttle between nucleus and PM via nuclear import pathway by importin α/β proteins [9]. In the nucleus hPLSCR1 binds to the inositol 1,4,5 triphosphate receptor type 1 (IP3R1) and up regulates its expression [10]. In addition, hPLSCR1 also interacts with topoisomerase and enhances the decatenation activity [11]. hPLSCR1 has been shown to regulate the differentiation and proliferation of cells by growth factor stimulation, phosphorylation of PLSCR1 by Src-kinase translocated it to the nucleus where it was shown to up regulate IP3R1 gene expression [12]. PLSCR1 is also substrate for other kinases such as IgE receptor tyrosine kinase, c-Abl kinase indicating it functions as a signaling molecule involved in

Abbreviations: hPLSCR1, human phospholipid scramblase 1; TFs, transcription factors; ChIP, chromatin immunoprecipitation; EMT, epithelial mesenchymal transition; PLs, phospholipids; PS, phosphatidylserine; RLU/β-gal, relative luciferase activity/β-galactosidase activity.

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diverse signaling pathways [13]. Interferon has been shown to up regulate hPLSCR1 expression and is considered as an amplifying factor in antiviral responses [14]. hPLSCR1 has also been shown to interact with multiple other proteins which include onzin, ECM1, HCV proteins EI and E2, CD1 and 4 cellular receptors thereby regulating their functions [15–18].

In variety of cancer cells variable expression of hPLSCR1 was observed notably in myeloid leukemia where its expression was down regulated [19]. In the myeloid leukemic U937 cells inducible expression of PLSCR1 arrested the proliferation of cells at G1 phase implicating the antileukemic role of PLSCR1. Induction of PLSCR1 increased expression of cyclin dependent kinase proteins such as p27 and p21 and down regulation of S-phase proteins such as SKP2. Antiapoptotic proteins such as c-myc and Bcl-2 were also down regulated upon PLSCR1 induction [19]. However despite the progress made in understanding the antileukemic role of hPLSCR1, the exact mechanism by which PLSCR1 down regulation occurs in such cancer cells remains unknown. In this study, we hypothesize that Snail TF might bind to promoter region of hPLSCR1 and regulate its expression based on bioinformatic studies. Our results clearly showed that Snail transcription factor binds to PLSCR1 promoter and down regulates its expression suggesting its possible implications in cancer therapy.

2. Materials and methods

2.1. Cell culture

Hek-293, IMR-32 cells were maintained in DMEM and RPMI-160 (Hyclone, Logan, UT, USA) medium supplemented with 10% and 20% FBS respectively and antibiotics at 37 °C and 5% CO_2. O-Nitrophenyl-β-D-galactopyranoside (ONPG), Adenosine triphosphate (ATP) and D-luciferin were obtained from Sigma Aldrich (USA).

2.2. Plasmid construction

Snail transcription factor cDNA was directionally cloned between BamH1 and Xho1 sites of pCDNA vector which contains N-terminal FLAG tag. Sequencing was done to verify the construct; expression of the construct was probed by Western blotting (anti-FLAG tag). For generating pGL3-hPLSCR1 promoter construct, a ~1.5 kb fragment of PLSCR1 promoter ranging from +25 to −1500 bp was PCR amplified from human genomic DNA using Phusion High Fidelity polymerase (Finnzymes, Finland) with following forward primer 5″-ATATATGGTACCCAGTCACCATTGGCGGCAC-ATTTCAG-3″ and reverse primer 5″-ACATCTCTCGAGGAGACTC-CAGAGACGTTTGTCCGGTG-3′ and cloned directionally between *Kpn1* and *Xho1* sites of pGL3 basic vector (Promega, Madison, USA) and the construct was verified by sequencing. Deletion constructs were generated by deletion the promoter from −1525 site using the following forward primer 5″-ATATATGGTACCCCGTCT-GCCACCCGGCCCTG-3″ for -1244 deletion, 5″-ATATATGGTACCG-TCCGTACTAGAAGAGGATCAATTGC-3″ forward primer for −990 deletion and 5″-ATATATGGTACCGCTGCTCTTGCCCCAAGGGGGG-3″ forward primer for −720 deletion and the same reverse primer used to clone full promoter was also used to generate deletion constructs. Deletion constructs generated contained at least one TATA box from the transcription start site. Point mutation within the putative Snail binding site was generated with the following forward primer 5″-GACCCATTTGTGAGTAAGTTG*GGTG*AATCGAT-TTTGTCCG-3″ and reverse primer 5″-CGGACAAAATCGATTCACC-CAACTTACTCACAAATGGGTC-3″ using site directed mutagenesis (SDM) kit (Stratagene, La Jolla, USA). PCR set up for SDM was as follows: 50 μl PCR reaction mixture was set up containing 5 μl 10× PCR buffer, 2.5 μl F-primer (10 pmol/μl), 2.5 μl R-primer (10 pmol/μl), 0.3 μl template, 1 μl (10 mMdNTP's), 0.5 μl Pfu Ultra polymerase (Thermo Scientific) and 38.2 μl of water. PCR amplification included initial denaturation at 95 °C for 5 min, the subsequent cycles (18) included denaturation at 95 °C for 50 s, annealing at 60 °C for 50 s and extension for 7 min at 72 °C. Final extension was carried for 10 min at 68 °C. PCR product was then *DpnI* digested for 1 h and transformed into DH5α *Escherichia coli* cells. Positive clones and point mutation was and confirmed by nucleotide sequencing.

2.3. Transfection and luciferase assay

HEK cells (60–70% confluent) were transfected with respective constructs along with Snail TF using Transpass D2 transfection reagent (NEB, UK) as per manufacturer's instruction. 24 h post transfection cells were washed with PBS, lysed and luciferase assay was performed using the assay buffer containing 1 mM luciferin salt, 3 mM ATP, 15 mM $MgSO_4$ and 30 mM Hepes-7.8. β-Galactosidase gene was co-transfected as internal control to normalize errors due to differences in transfection efficiency. β-Galactosidase assay was performed using ONPG as substrate and the absorbance was measured at 420 nm.

2.4. Western blot

Transfected cells were lysed in lysis buffer (50 mM Tris-7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM PMSF and protease inhibitors). Total protein was estimated by BCA method using BSA as standard. 50 μg of total protein was loaded onto 12% SDS–PAGE and was transferred onto nitrocellulose membrane. Membrane was blocked using blocking buffer (10 mM Tris-7.4, 150 mM NaCl, 3% BSA and 0.1% Tween-20 for 1 h at room temperature. Immunoblotting was done using anti-PLSCR1 (Santa Cruz), anti-FLAG (Santa Cruz) and anti-actin (Sigma).

2.5. shRNA mediated Snail knockdown

Snail shRNA (sc-38398) and control shRNA (sc-108060) plasmids were purchased from Santa Cruz Biotechnology Inc. (USA). IMR-32 cells were grown till 60–70% confluence and were transfected with respective Snail-shRNA/control sh-RNA plasmids. Post transfection, cells were lysed and total RNA was isolated using RNAeasy kit (Qiagen) according to manufacturers protocol. RNA was quantified and was reverse transcribed to create cDNA using TaqMan Universal master mix (NEB). Quantitative PCR was performed in 96 well plate using SYBR master mix on a Real time detection system (Eppendorf) using the following PLSCR1 gene specific primers, forward 5′-CTG ACT TCT GAG AAG GTT GC-3′ and reverse, 5′-GAA TGC TGT CGG TGG ATA CTG-3′. PLSCR1 mRNA levels were normalized relative to actin expression. Additionally the expression levels of hPLSCR1 and Snail post knock down was analyzed by Western blot analysis.

2.6. Chromatin immuno precipitation

CHIP assays were performed as described earlier [20]. Subsequent to cell (IMR-32) lysis the genomic DNA was sheared into small fragments with sizes of ~400 bp using Branson 450 Sonifier. DNA–protein complexes were pulled down using Snail antibody (2 μg) over night at 4 °C, normal rabbit IgG antibody was used as a control. Post protease treatment the DNA was recovered and subjected to 33 cycles of PCR using forward primer 5″-TCTCAGCT-GATGCATGATCACCTATCTTA-3″ and reverse primer 5″-ATCTCAG-GATATCCCACCTTGAGTTTAATCCG-3″ corresponding to −1217 to

−940 region of promoter (~250 bp). PCR products were resolved on 0.8% agarose gels and visualized using ethidium bromide. Snail pulled down PCR fragment was then gel excised and sequenced, the sequencing result was then analyzed using NCBI (BLAST).

2.7. Statistical analysis

All the experiments were performed at least three times. Paired two-tailed students *t*-test was used to determine the statistical significance of difference between 2 groups. $p < 0.05$ were considered to be statistically significant.

3. Results

3.1. Identification of transcriptional factor binding sites

To identify the cis-acting elements within the hPLSCR1 promoter, preliminary screening was done using ConSite, open source software that predicts the binding sites for known TFs. Fig. 1A shows the putative TFs binding sites within the hPLSCR1 promoter region which include Snail, AML-1 and Sox-5. The identified putative TFs binding sites by ConSite were performed using a cut of score of 100%. The putative binding site for the Snail TF within the promoter was CAGGTG which was similar to the Snail consensus binding site CANNTG. Hence, a 1523 bp region corresponding to the 5′-flanking region of hPLSCR1 including the transcription start site was PCR amplified and cloned into pGL3 basic vector. The effect of Snail TFs on expression levels of hPLSCR1 was studied by co-transfecting it with various promoter constructs. To confirm the expression of Snail TF in various cell lines used, Western blot analysis was performed using anti-FLAG antibody since the Snail TF was cloned downstream of a FLAG tag and actin as control (Fig. 1B). Additionally to overcome variable transfection efficiencies, β-galactosidase was co-transfected and β-galactosidase activity was used to normalize the data.

3.2. Snail down regulates hPLSCR1 expression

To study the effect of Snail TF on hPLSCR1 expression, transient co-transfection experiments were performed. Fig. 2A shows a dose dependent decrease in hPLSCR1 expression in HEK-293 cells, with increasing concentrations of transfected Snail TF. Dose dependent expression of Snail co-transfection was quantified by Western blot using anti-snail antibody (Fig. 2A). Snail mediated down regulation of hPLSCR1 promoter was further verified at translation levels by Western blot using anti-hPLSCR1. Fig. 2B confirms the decrease in hPLSCR1 protein levels with increasing concentrations of Snail TF. To further confirm the down regulation of hPLSCR1 by Snail, we used anti-snail shRNA and checked for its effect on hPLSCR1 expression. An increase (>2.5-fold) in hPLSCR1 mRNA levels was observed in cells transfected with Snail shRNA compared to control shRNA (Fig. 2C). Effectiveness of Snail shRNA knockdown was further assessed by Western blot. Snail shRNA knockdown significantly decreased Snail expression with an increase in hPLSCR1 expression levels (Fig. 2D). These results confirm that Snail mediated down regulation of hPLSCR1 is specific.

3.3. Deletion constructs of Snail binding site retained hPLSCR1 promoter activity

To further identify whether Snail TFs down regulates hPLSCR1 expression by binding to its promoter, deletion constructs were generated and luciferase assays were performed. Generated deletion constructs −Δ1244, −Δ990 and −Δ720 contained at least one TATA box and all the deletion constructs lacked predicted Snail TF binding site (Fig. 3A). It can be seen that there was ~4-fold increase in promoter activity for −Δ1244 deletion construct and greater than ~10-fold increase in promoter activity for −Δ900 and −Δ770 deletion constructs respectively when compared to full length hPLSCR1 promoter (Fig. 3B).

3.4. Confirmation of Snail binding site

Bioinformatic studies revealed that putative Snail transcription factor binding site was found within the regulatory region of the promoter (−1123 to −1117) and is in agreement with our results that −Δ1244 construct regained hPLSCR1 promoter activity (Fig. 3C). In order to nail down the exact binding site, point mutations were performed in wild type (CAGGTG) to (ATGGTG). Our results clearly showed that the predicted Snail binding site is indeed true Snail binding site as evident from ~40% increase in mutant promoter activity of hPLSCR1 when compared to wild type.

3.5. ChIP assay

ChIP was used to further confirm the interaction of Snail with endogenous hPLSCR1 promoter. Chromatin fragments bound with transcription factor were prepared from IMR-32 cells. Anti-Snail antibody was then used to immunoprecipitate Snail bound chromatin fragments followed by PCR with primers corresponding to −1217 to −940 (~254 bp) region of hPLSCR1 promoter. Anti-IgG antibody was used as a negative control in order to show that Snail binding was specific. Fig. 4 shows PCR amplified products using

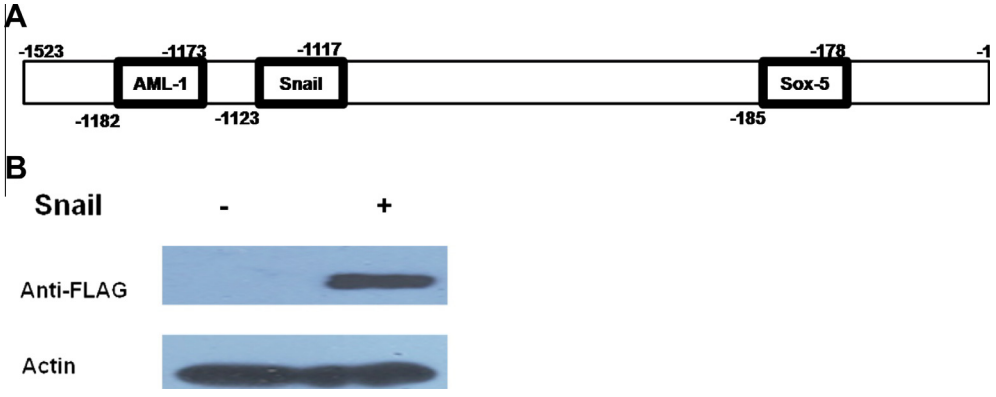

Fig. 1. TFs binding site and Snail expression. (A) Schematic showing binding of various TF's which bind to hPLSCR1 promoter. ConSite a TF search tool was used to screen the putative TF binding site based on the consensus binding sequences of known TFs. (B) Validation of Snail overexpression in HEK-293 cells by Western blot using anti-FLAG antibody.

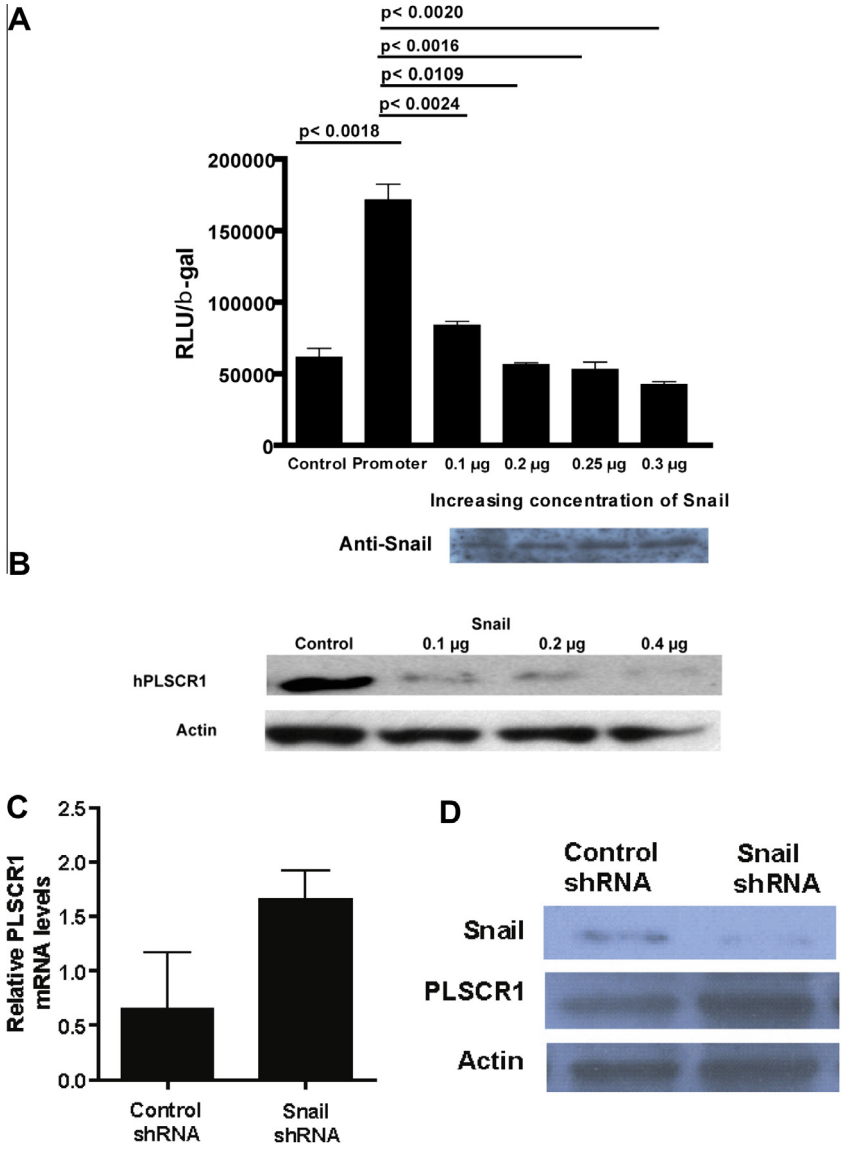

Fig. 2. Snail mediated down regulation of hPLSCR1 and knock down of Snail. (A) Luciferase assay showing dose dependent decrease of hPLSCR1 promoter activity in HEK cells transfected with increasing concentration of Snail TF, luciferase values expressed were normalized with respect to β-galactosidase value; inset – Western blot showing dose dependence increase in Snail expression using anti-Snail antibody (B) Western blot showing endogenous expression of hPLSCR1 in HEK cells transfected with Snail TF, expression was probed with Anti-hPLSCR1. (C) Knock down of Snail, IMR-32 cells were transfected with control shRNA and Snail specific shRNA. Post-transfection, mRNA levels of hPLSCR1 in cells expressing indicated shRNA were analyzed by RT-PCR. (D) Western blot showing expression levels of Snail and hPLSCR1 post shRNA knock down. Data shown are means ± S.E. (error bars) form three separate experiments.

Anti-Snail and Anti-IgG pulled down chromatin fragments. Sequence of ChIP amplified PCR fragment was further verified by sequencing and the sequenced data matched the 5′-flanking region of the hPLSCR1 promoter confirming specificity.

4. Discussion

hPLSCR1 a multifunctional protein shows variable expression in a variety of cancers. In ovarian cancer cell line HEY 1B, increased expression of hPLSCR1 was observed to enhance the invasive properties in ovarian cancer cells [21]. It has also been reported that over expression of hPLSCR1 in athymic nude mice was associated with marked suppression of tumor development, increased infiltration of macrophages and also promoted differentiation. In the myeloid leukemia cell line U937, over expression of hPLSCR1 arrested proliferation of cells at the G1 phase. Additionally the cells also underwent granulocyte like differentiation and was also found to be sensitive to etoposide induced apoptosis [19]. In the malignant adenocarcinoma hPLSCR1 was found to be over expressed compared to normal colorectal mucosa and is now used a biomarker for colorectal cancer diagnosis [22]. These data indicate that hPLSCR1 is regulated by diverse regulatory pathways however till date the exact regulatory mechanism of hPLSCR1 notably transcription regulation is unknown. TFs have been shown to play a major role in the cancer progression notably the TFs such as NF-kappa B, AP-1, ATF, ETS and Snail [23]. The first identified member of the Snail super family is Snail and was found to be essential for the formation of mesoderm [24]. These TFs are zinc finger type of TFs which corresponds to C_2H_2 type and typically function as transcriptional repressors. Consensus binding site for Snail TFs is a core of six bases CAGGTG and are identical to E-box which is the binding site for basic helix-loop-helix TFs. Since its identification, several reports have shown the central role played by Snail TFs in

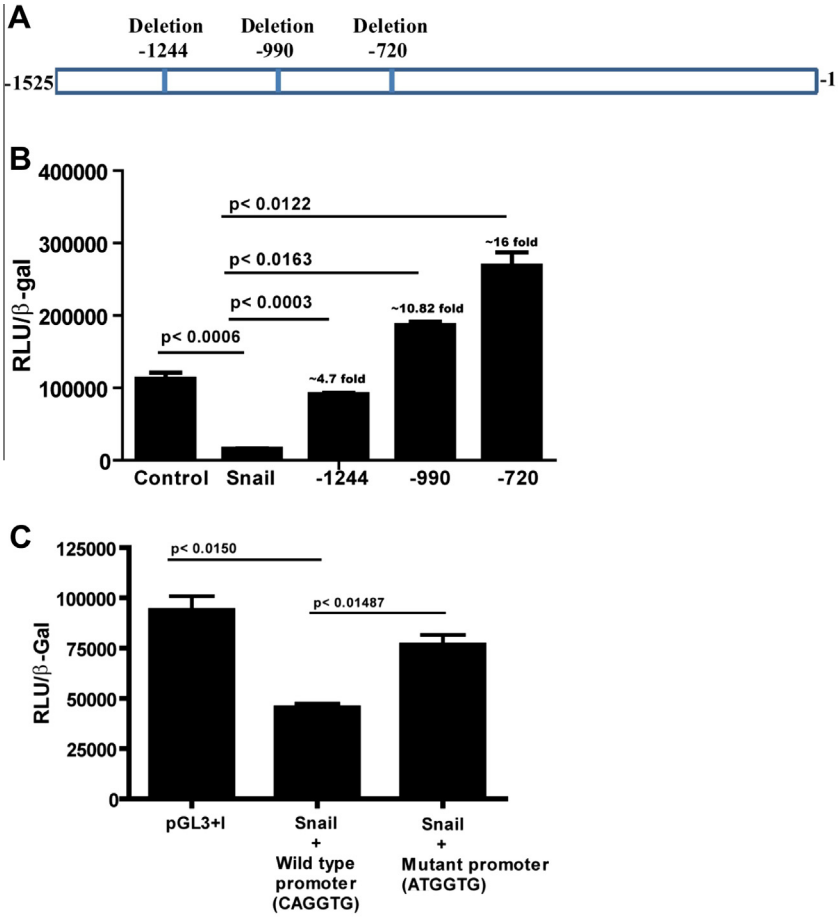

Fig. 3. Identification of Snail binding site. (A) Schematic showing various deletion constructs generated to map Snail binding site. (B) Luciferase assay (RLU/β-gal) with various deletion constructs of hPLSCR1 promoter co-transfected with Snail TF. (C) Luciferase assay (RLU/β-gal) with point mutation in the predicted Snail binding site of hPLSCR1 promoter in presence of Snail TF. Data shown are means ± S.E. (error bars) form three separate experiments.

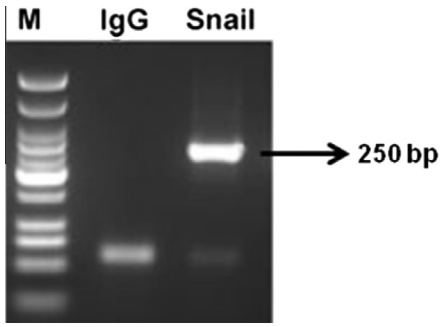

Fig. 4. CHIP assay. Chromatin fragments were prepared from neuroblastoma IMR-32 cell line and were subsequently used for immune precipitation with rabbit IgG and Snail specific antibody. A ~250 bp internal region of hPLSCR1 promoter was used to confirm Snail binding to hPLSCR1 promoter.

cancer progression. Snail has been implicated in the regulation of a number of genes such as IL-8, TGF-β-3, maspin tumor gene and p63 [4,25,26]. Recent report has also shown that Snail TF regulates the expression of its own promoter and the Snail gene expression is regulated via ERK2 and PI3K signaling pathways [27]. The expression levels of hPLSCR1 in a variety of cancers are inversely related to progression of carcinoma, thereby implying that it has diagnostic and prognostic values for cancer progression. The presence of Snail binding site within hPLSCR1 promoter and its down regulation in a variety of cancers led us to study whether Snail TF regulates hPLSCR1 expression. Since hPLSCR1 is expressed in a wide variety of tissues we used HEK-293 and IMR-32 cells to confirm the interaction of Snail with hPLSCR1.

Preliminary study of the hPLSCR1 promoter using bioinformatic tools revealed the presence of binding sites for AML-1 Sox-5 and Snail TFs. Since Snail TFs is a key molecule central for the progression of cancer it was evaluated for its effect on hPLSCR1 expression. In presence of Snail TF, there was significant reduction of hPLSCR1 expression in a dose dependent manner. Down regulation of hPLSCR1 by Snail may be significant since Snail is known to promote withdrawal of apoptotic signals and hPLSCR1 being a crucial effector of the apoptotic cascade [28]. Cancer cells are known to evade apoptosis by preventing exposure of phosphatidylserine (PS) and transbilayer movement of PS from inner leaflet to outer leaflet has been shown to be facilitated by hPLSCR1 [8]. To determine the binding site of Snail within hPLSCR1, deletion constructs were generated and luciferase assays were performed. Results confirmed the binding region of Snail to be within the −1525 to −1244 which was within the similar region predicted by ConSite to contain the Snail binding site.

The promoter activity of full length hPLSCR1 in the absence of externally transfected Snail TF was higher than the Snail transfected cells (Fig. 3B). This could be explained by the fact that the net expression of the promoter is a result of combinatorial effect of stimulatory and down regulating TFs and hPLSCR1 promoter contains putative binding site for several other TFs such as c-FOS, Sox-5, HNF-3beta and AML-1. Also regulation of gene expression by TFs depends on their cellular concentration, activity and their interaction with other

TFs/RNA polymerase II [29]. High levels of Snail TF is generally observed in cancer cells compared to normal cells and it could be possible that due to low levels of Snail TF in HEK-293 cells it does not show promoter repression [30]. Knock down studies using Snail specific shRNA further confirms that Snail mediated transcriptional regulation of hPLSCR1 is specific. Point mutation confirms that −1117 to −1123 is the Snail binding site within the hPLSCR1 promoter. ChIP assay using neuroblastoma cell line also confirms that Snail TF binds to endogenous hPLSCR1 promoter. It should be noted that using 100% cut off score for ConSite analysis only one Snail binding site could be identified, screening using low cut off score revealed multiple binding site for Snail TF. However a detailed analysis of multiple Snail binding sites was not done and we hypothesize that they may display a combinatorial effect.

5. Conclusions

To conclude we show for the first time transcription regulation of hPLSCR1 by Snail TF and also confirmed the predicted Snail binding site within hPLSCR1 promoter. Interaction of Snail with hPLSCR1 promoter may have profound impact in variety of cellular processes notably as to how cancer cells escape apoptosis.

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