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ANTINEOPLASTIC AND PROTEOMIC SIGNATURE OF SIMVASTATIN TREATED CD44$^+$/ CD24$^-$ TRIPLE NEGATIVE BREAST CANCER STEM -LIKE CELLS

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ABSTRACT

Simvastatin, a potent HMG CoA reductase inhibitor is gaining significant importance in targeting cancer stem cells. Despites its effects in reducing the expression of CSC surface markers and sphere forming ability in cancer stem cells their molecular mechanism remains poorly understood.In this study we employed label free quantitative proteomic profiling using mass tandem spectroscopy to corroborate the effect of Simvastatin on the cell proliferation, mammosphere formation and its apoptotic effect on CD44$^+$/CD24$^-$ Breast Cancer Stem Cells (BCSCs).Simvastatin induces inhibition of cell proliferation in CD44$^+$ CD24$^-$ BCSCs. Half inhibitory concentration IC_{50} was found to be 5 µg/ml. Further Simvastatin induces apoptosis in BCSCs stained with Nexin reagent. Furthermore Simvastatin treatment for 48 hrs with various concentrations significantly inhibited the growth of 6 days old mammospheres in a dose dependent manner. In label free quantitative proteomic profiling of Simvastatin treated and untreated BCSCs, 99 differentially expressed proteins were identified of which, 48 were upregulated and 51 were down regulated. Gene ontology and KEGG pathway enrichment analysis exposed 18 potential pathways associated with Simvastatin treatment. These identified pathways were shown to be related with carcinogenesis, tumor progression, metastasis, focal adhesion and metabolic effects in cancer cells. Among the down regulated proteins Vinculin, Heat shock protein beta-1(HSPB1) was significantly down regulated.Our study reveals that Simvastatin potentially targets BCSCs and their regulatory pathways. Further our proteomics profiling implies the better use of existing drugs and new targets in cancer therapeutics.

Keywords: ***Simvastatin, Breast cancer stem cells, Apoptosis, Proteomics***

INTRODUCTION

Breast carcinoma is the leading cause of cancer mortality among women and accounts for about 25% of all cancer in women.[1] Chemotherapeutic drugs, which target the reduction of tumor mass in the active phase of disease, fail to curtail the relapse of disease following remission.[2] The relapse is due to a small group of cells known as cancer stem cells (CSCs). These cells -Cancer stem like cells or Tumor initiating cells, have been demonstrated to possess tumor forming and self renewal properties.[3] In addition, it has been shown to be resistant to chemotherapy and causes widespread recurrence of metastasis.[4] Thus the drugs which selectively target cancer stem cells hold greater promise in the treatment of cancer. Simvastatin, an analogue of lovastatin ia a potent competitive inhibitor of 3-hydroxy-3-methylglutaryl coenzymeA reductase (HMG-CoA) is a well tolerated lipid lowering agent, which has recently gained importance in cancer therapeutics as it targets both the CSCs and neoplastic cells. Invitro and preclinical studies have clearly demonstrated the role of statins in inhibiting cancer cell proliferation & angiogenesis and induces apoptosis.[5] These effects are shown to be

associated with the depletion of downstream products of mevalonate pathways.[6] Further, clinical studies have shown the advantage of cancer prevention in patients receiving statins for correction of hyperlipidaemia.[7,8] Recently, it has been shown that statins reduce the expression of surface markers of cancer stem cells via transcriptional mechanisms, unrelated to HMG-CoA reductase[9] suggesting the pleotropic effects of statins. Simvastatin has been shown to reduce the CSC in drug resistance tumor cells and the sphere forming capacity in canine breast carcinoma cell line.[10] Although, Simvastatin was shown to target CSCs, its exact molecular mechanism of action, related pathways and targets remain poorly understood. To get a more comprehensive understanding of the actions of Simvastatin on CSCs and to find new targets, we employed the label free quantitative proteomic profiling by using mass tandem spectroscopy, an effective tool in cancer research to study the mechanism of drugs at its functional level.In the current study, we investigated the role of Simvastatin on the cell proliferation, apoptotic and mammosphere formation among $CD44^+$/ $CD24^-$ sorted cells of MDA-MB-231 cells in *vitro*, and further we explored the underlying molecular mechanism of Simvastatin employing label free quantitation proteomic profiling by tandem mass spectroscopy . We further identified differentially expressed proteins and potential signaling pathways in breast cancer stem cells (BCSCs) after Simvastatin treatment. The findings reported in this study strongly imply the role of Simvastatin in targeting BCSCs and its regulatory pathways.

MATERIALS AND METHODS

Cell culture and reagents

MDA-MB-231 human breast cancer cells were gifted by Yenepoya Research Centre, Mangalore. The cells were tested for mycoplasma contamination (Mycoplasm Kit, LONZA, USA) and cultured with DMEM supplemented with 10% FBS (Hyclone,UT,USA) and penicillin-streptomycin solution(Hyclone) and cultured at 37° C at 5% Co_2 incubator (Thermo scientific). For mammosphere culture, DMEM/F12 (GIBCO), epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), ITS AND B27 were purchased from Sigma, USA.For Label Free Quantitative (LFQ) proteomic analysis, Phosphate buffered saline ((PBS), Sodium Dodecyl Sulphate (SDS) and Urea were purchased from Ranchem, India. Ammonium bi-carbonate (ABC), Acetonitrile (CAN), Dithiothreitol (DDT), Iodoacetamide (IAA) & Trifluoroaceticacid (TFA) were obtained from Sigma Aldrich, USA. Trypsin-Protease was purchased from Pierce (Thermo Scientific).

Flow cytometry

MDA-MB-231 cells were grown to 80–90% confluence prior to flow sorting. The cells were trypsinized for dissociation, washed twice with FACS buffer (PBS containing 1% BSA and 1mM EDTA), and resuspended in FACS buffer. Direct-labeled antibodies, anti-CD24- PerCP/Cy5.5 (BD Bioscience, Pharmingen) and anti-CD44 Alexa Fluor® 488(Biolegend, USA) , were added to the sample and incubated in the dark for 20 min on ice. Subsequently, cells were washed twice with FACS buffer and resuspended in FACS buffer followed by flow cytometry using S3e™ Cell Sorter (BIO-RAD). Side scatter and forward scatter profiles were used to eliminate cell doublets. Cells were reanalyzed for high purity.

Activation of Simvastatin

Simvastatin lactone (Sigma, USA) was activated to its acidic form before the cell culture experiment. Briefly, 8 mg of Simvastatin was dissolved in 0.2 ml of 100% ethanol, with subsequent addition of 0.3 ml of 0.1 N NaOH. The solution was heated at 50°C for 2 h in a dry bath and neutralized with HCl to pH 7.2. The resulting solution was brought to a final volume (1 ml) with distilled water.

Mammosphere culture

Mammospheres were cultured in suspension (1000 cells/ml) in serum-free DMEM/F12 media(1:1), supplemented with B27 , 10 ng/ml EGF, 20 ng/ml bFGF and ITS in ultra low attachment plates as described previously.[11] The growth was examined by adding Simvastatin at various concentrations to 6-day old mammospheres and the number of mammospheres where counted after 48 hrs of treatment and represented as Mean± SD of triplicates.

Cell proliferation assay

The $CD44^+$/ $CD24^-$ sorted cells were cultured in a 96 well plate at a density of 1×10^4 cells/ 100 µl/ well in DMEM/F12 (1:1) supplemented with 10 ng/ml b-FGF, 20 ng/ml EGF ,ITS,B27 in order to maintain the stemness. After overnight incubation, the cells were treated with different concentration of Simvastatin for 48 hrs. During the last four

hours, MTT reagent (5mg/ml) was added to each well. The production of MTT –formazan crystals was dissolved in 100 µl of DMSO and absorbance was measured at 560/ 620 nm.

Cell apoptosis study

The $CD44^+$/ $CD24^-$ sorted cells were seeded in 35-mm dishes at 1 x 10^5 cells/dish and treated with either different concentrations of Simvastatin or vehicle for 48 hrs. The cells were stained with Guava Nexin reagent (Millipore, USA) and assay was carried out according to the manufacturer's instructions. Apoptosis was detected using a Guava EasyCyte Flow Cytometer (Merck Millpore,USA) and analyzed using FCS Express 6 plus research edition software.

Protein Precipitation

For proteomic profiling, $CD44^+$/ $CD24^-$ MDA-MB-231 cells were cultured in serum free media and treated with 5 µg/ ml of Simvastatin for 24 hrs. Treated and untreated cells were suspended in lysis buffer (4% SDS + 50 mM TEABC) and sonicated in ice , heat blocked at 90° C, centrifuged at 12,000 rpm , supernatant was collected and stored at -20° C till use. The protein concentration was measured by Bradford assay for normalization. 30 µg protein each from the control and treated samples were subjected to acetone precipitation to remove salts and other interfering substances. The protein precipitate was dissolved in urea and the samples were processed immediately for trypsin digestion.

Reduction, Alkylation, Trypsin Digestion and Sample Clean up

The protein pellets from each sample were dissolved by adding 10 µL of 6 M urea and the volume was brought to 15 µL with HPLC grade water. Samples were then reduced by addition of 1.5 µL of 100 mM DTT and heated at 90° C for 10 min, brought to room temperature and the reduced -SH groups were alkylated by adding 1.5 µL of 200 mM IAA and incubated in the dark at RT for 15 min. 90 µL of ABC was added to the sample and the proteins were digested by adding 1 µL of 1 mg/ml trypsin protease and incubating at 37 °C for 16 hours. The reaction was stopped by addition of 2 µL of concentrated TFA. The digested peptides were dissolved in 0.1% TFA, 5% ACN in water for MS-analysis.

Mass spectrometry analysis of proteins

HPLC-CHIP-MS

Agilent 1260 infinity HPLC-Chip/MS system is a microfluidic chip-based technology that incorporates peptide enrichment and separation and provides high-sensitive nano-spray. Charged peptides from HPLC-Chip system were directly infused into mass-spectrometer for detection. Following HPLC-Chip-MS conditions were used for acquiring the MS and MS/MS spectrum of the peptides. Chip ID:G4240-62030 Chip Name: High Performance Chip, 360 nanoliter enrichment column, 150 mm X 75 µm separation ,column Solvent A: 0.1% Formic Acid ,Solvent B:90% ACN / 10% (0.1% Formic Acid), Flow Rate: 0.3 µl / min Run Time:120 minutes, Sample Volume: 5 µl ,MS Scan Range: 275 to 1700 m/z ,MS Scan Rate: 8 spectra / sec, MS/MS Scan Rate: 3 spectra / sec Ion Polarity:Positive Ions Fragmentor Voltage:170 V, Skimmer Voltage:65 V ,Octopole RF Voltage:750V Gas ,Temperature: 250°C& Drying Gas: 5 L / min

Bio-informatics analysis of data

Protein Identification was performed with the following criteria: (a) Trypsin digested peptides with 4 missed cleavages allowed, (b) peptide tolerance < 50 ppm, (c) > 2 unique peptides, (d) FDR < 5%. Fasta files for human proteins was downloaded from the uniprot database. For the analysis, proteins identified in at least 2 out the three replicates in each group were considered. Thus, a list of identified proteins was generated. Sum of Unique peptide intensity was used for semi-quantitative analysis. Ratio was calculated for proteins identified in both the treatment and control groups. Ratio of >1.5 was considered as "up-regulated" and ratio < 0.5 was considered as down-regulated. The signaling pathways, Gene ontology and interaction network were analyzed using open source STRING (version 10) program (www.string-db.org).

Western blot analysis

Cell lysates were collected using lysis buffer supplemented with Protease inhibitors and immunodetection of electrophoresis. Resolving was performed using standard protocol. The HSPB1 and Vinculin antibodies were obtained from abcam, USA. Immunodetection was accomplished using chemiluminescence and data were acquired using digital image system(Bio-rad) . The bands were analysed and represented using Image J software.

STATISTICAL ANALYSIS

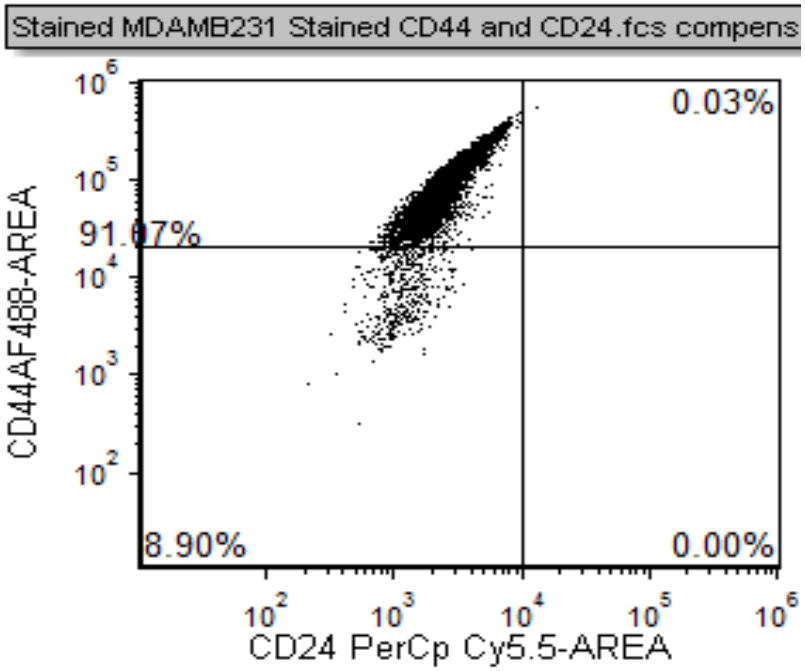
The numerical data were represented as mean ± standard deviation. Student's t test or ANOVA was used to determine the difference between the groups using SPSS software version 20. The significance level was set at $p < 0.05$ for all tests.

RESULTS

Anti-cancer effect of Simvastatin in MDA-MB-231 CSC-like cells.

The breast cancer stem-like cells were isolated from MDA-MB-231 triple negative breast cancer lineage based on stemness markers. CD24, CD44 have been shown as surface markers for Breast CSCs. The cancer stem-like cells were therefore enriched by sorting for $CD44^+$ $CD24^-$ subpopulation. Flow cytometry was employed to investigate the expression and sorting of $CD44^+$ /$CD24^-$ subpopulation in MDA-MB-231 cells (Figure 1)

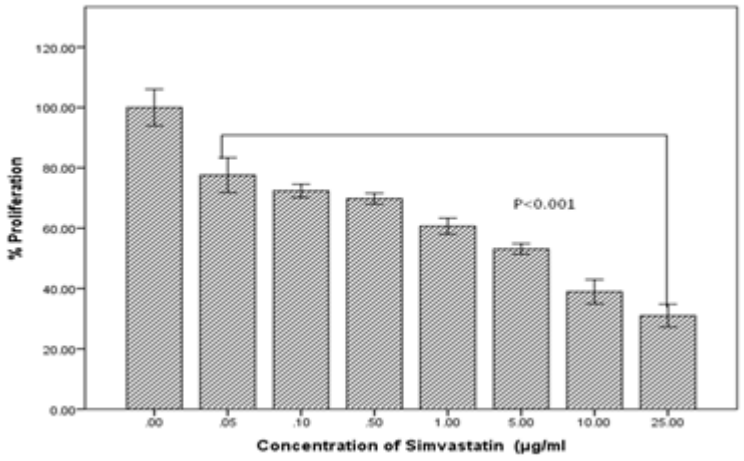
Figure 1
Flow cytometry analysis of CD24 and CD44 expression in MDA-MB-231cells.



Dot plots of CD44- AF488 against CD24-PerCpCy5.5. fluorescence

To explore the antiproliferative activity of Simvastatin in $CD44^+$ $CD24^-$ BCSCs, the cells were treated with different concentrations of Simvastatin for 48 hrs . Simvastatin induces inhibition of cell proliferation in BCSCs (Figure 2). Half inhibitory concentration IC_{50} was found to be 5 µg/ml and all subsequent experiments were carried out using 5 µg/ml of Simvastatin. Further Simvastatin induces apoptosis in BCSCs stained with Nexin reagent(Figure 3 A&B) and Simvastatin treatment for 48 hrs with various concentrations significantly inhibited the growth of 6 days old mammospheres in a dose dependent manner (Figure 4A&,B),

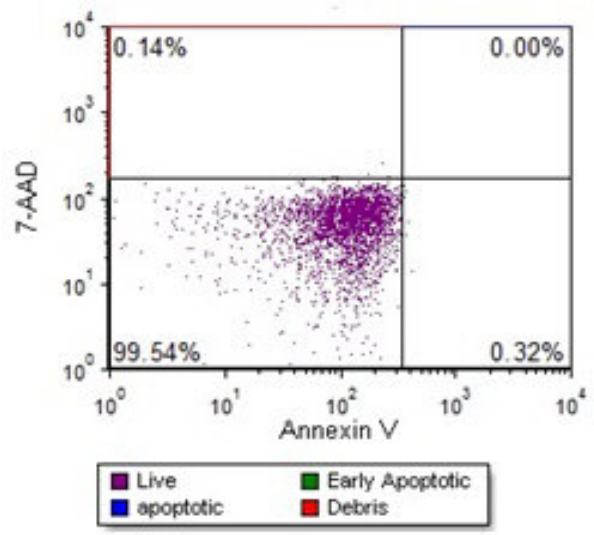
Figure 2
Simvastatin induces growth inhibition in BCSCs

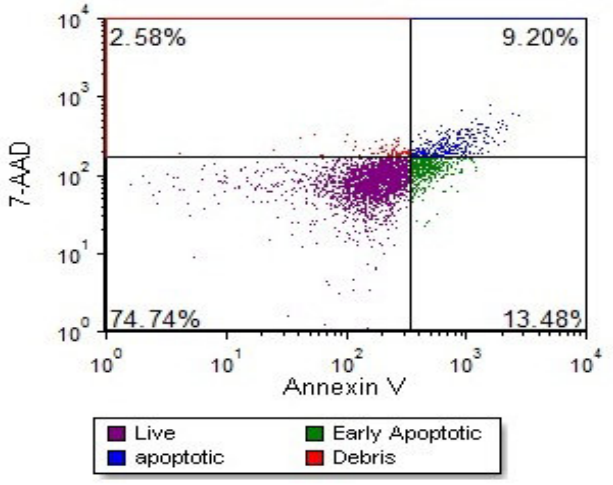


$CD44^{+}/CD24^{-}$ were seeded in 96-well plate and treated with increasing concentrations of simvastatin for 48 h. Cell proliferation was analyzed by measuring fluorescence at 620 nm after the addition MTT reagent. Data were presented as mean± standard deviation (n=3) p<0.001 compared with the control

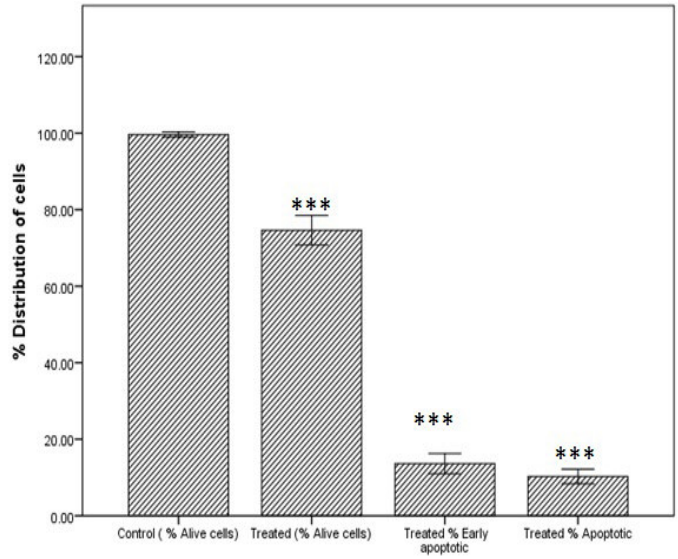
Figure 3
Simvastatin induces apoptosis in BCSCs.

A.





B.

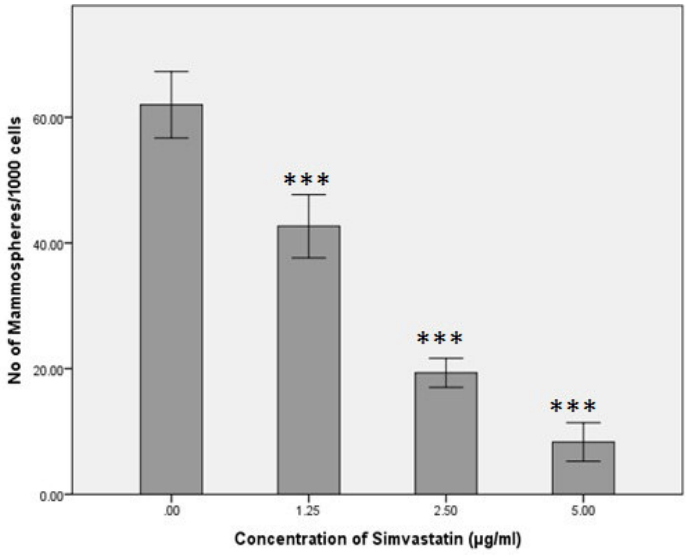


(A) CD44^{+}/CD24^{-} were seeded in 35-mm dishes and treated with simvastatin (5 µg/ml or vehicle) and cultured for 48 h. The cells are stained directly with Guava Nexin Reagent , a pre-made cocktail containing Annexin-PE and 7-AAD in buffer ,in a 200 µl final volume after a 20 –minutes incubation at room temperature, the samples are acquired on Guava system. (B) A bar graph represents the percentage distribution of cells in each related portion p<0.001.

Figure 4

Simvastatin inhibits mammosphere formation

A.



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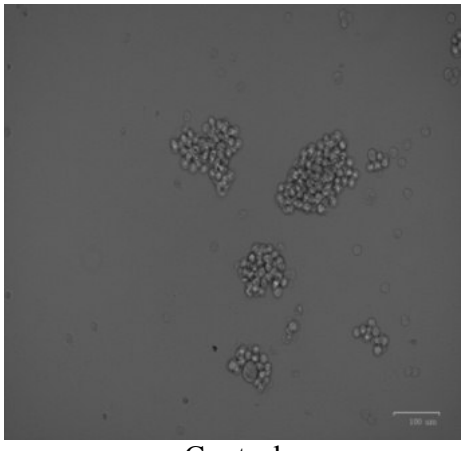
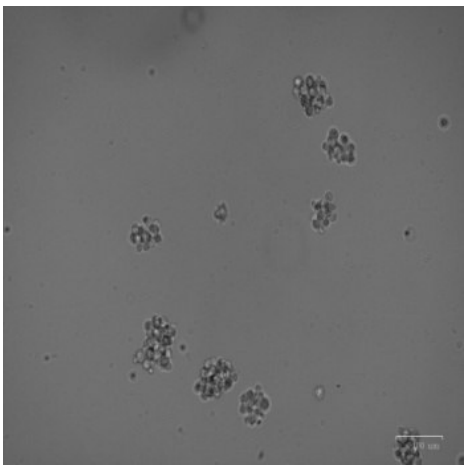
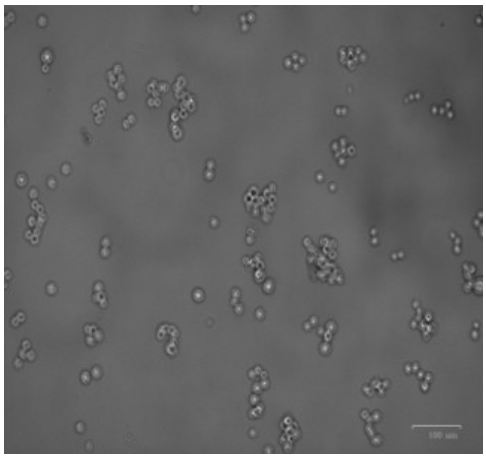


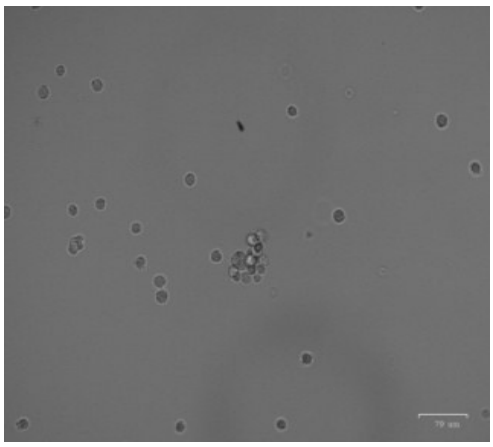
Control



1.25



2.5



5

(Concentration of Simvastatin in µg/ml)

(A) Effect of Simvastatin on number of mammospheres. Significant variation from the control were indicated by ***p <0.001, (B) An increase in the concentration of Simvastatin reduces the number and size of the mammospheres.

Overview of quantitative proteomics

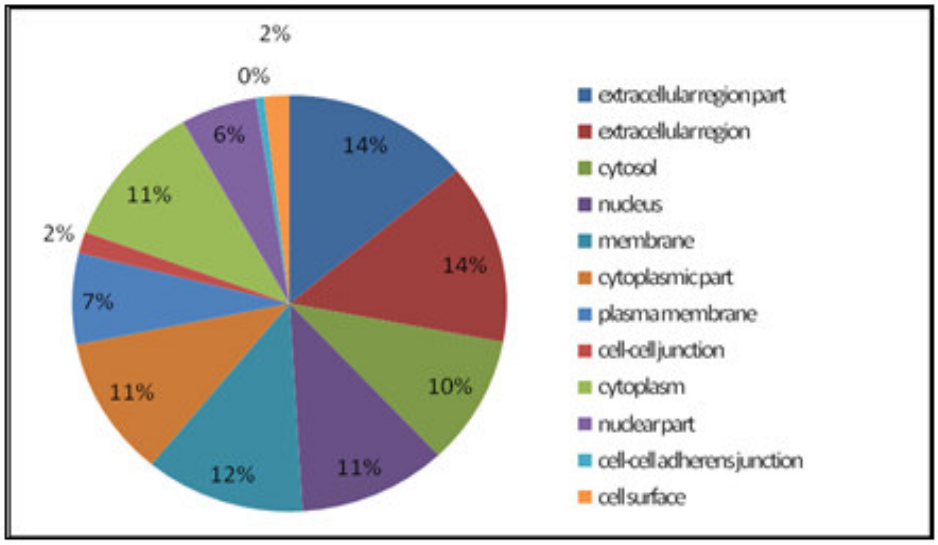
Label free quantitative proteomics was performed in protein precipitate extracted from $CD44^{+}$ $CD24^{-}$ BCSCs treated with and without Simvastatin , to understand the cellular and molecular mechanism of its anticancer activity at the protein level. Proteins were searched against human proteome database available from uniport. A filtering criteria of greater than 2 unique peptides with 5% FDR and a Q-value of zero was used. After data filtration 99

proteins showed significance difference in Simvastatin treated cells. Among the 99 differentially expressed proteins (DEPs), 48 proteins were up regulated proteins (Table 1) and 51 proteins were down regulated proteins (Table 2).The gene ontology analysis was performed using String database for all differentially regulated proteins for their molecular function, biological process and cellular component (Figure 5). Approximately 40% were binding proteins and remaining were catalytic and enzyme regulators.

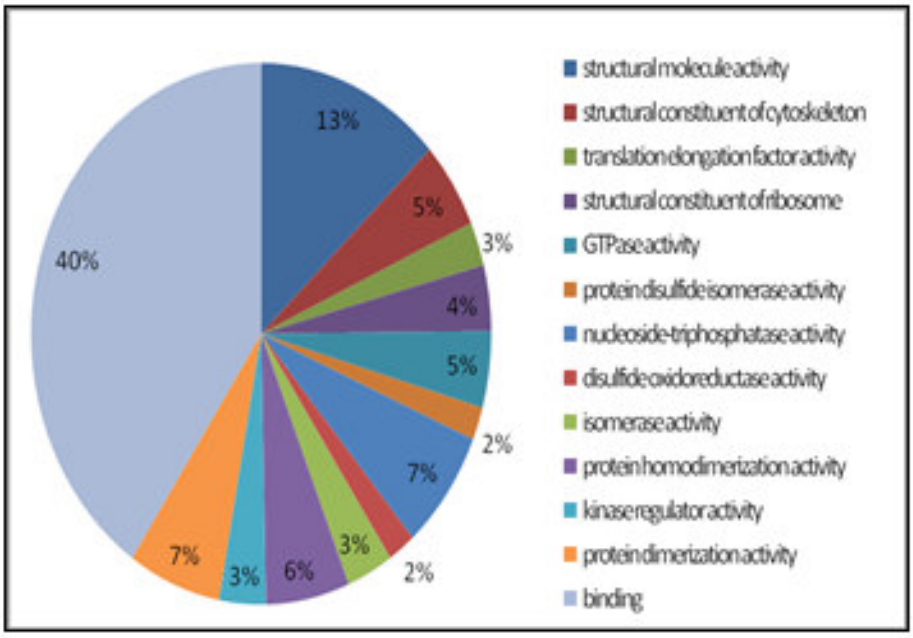
Figure 5

Categorization of all differentially expressed proteins by GO analysis

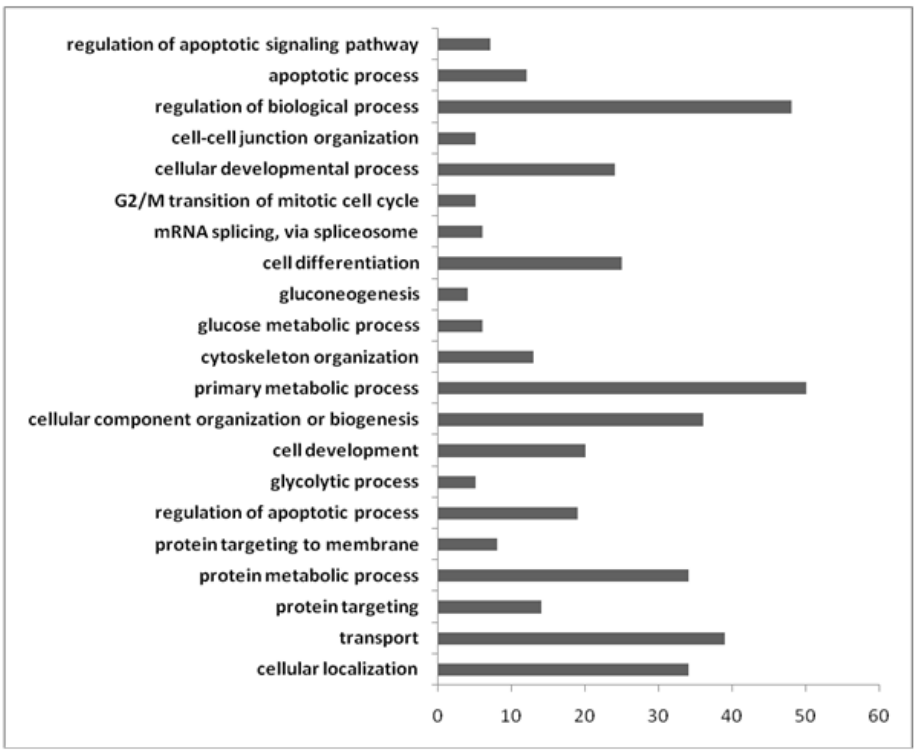
A.



B.



C.



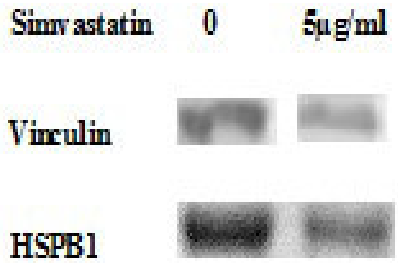
(A) Cellular component. (B) Molecular function. (C) Biological process (P<0.05)

KEGG pathway enrichment analysis was also performed based on DEPs. A total of 18 pathways were identified $p < 0.05$ (Table 3). This includes focal adhesion (Vinculin , Talin-1- P18206,Q9Y490), regulation of actin cytoskeleton (Cofilin-1- E9PK25), adherent junction (Alpha-actinin-1- P12814) , carbon metabolism (Fructose-bisphasphate aldolase- J3KPS3), Glycolysis/ Gluconeogenesis (Phosphoglycerate mutase-QOD2Q6), PI3K-Akt signaling (Heat shock protein beta-1, CDC37 protein -P14625,Q6FG59) and Hippo signaling pathway (Protein kinase c inhibitor protein -1 -P27348).

Validation of proteomic analysis

Based on the functional role of DEPs in relation to TNBCSCs , western blotting analysis was performed on some selected proteins (such as "HSPB1, Vinculin" which were significantly downregulated upon Simvastatin treatment) to further validate our LFQ results. Protein Vinculin has been shown to possess multiple roles in tumor progression such as , Epithelial to Mesenchymal Transition (EMT) , focal adhesion and multiple oncogenic signaling pathways and plays a vital role basal cell carcinoma. Heat shock protein beta-1 was down regulated by Simvastatin treatment and it is associated with multiple pathways in cancer (p53, MAPK & VEGF)and regulates apoptosis . Notably, the western blot images correlated very well with our LFQ data (Figure 6) .

Figure 6
Western Blotting images



The expression of Vinculin and HSPB1 in $CD44^{+}/CD24^{-}$ BCSCs treated with or without Simvastatin(5 µg/ml)

Table 1.
Annotation of up-regulated proteins after Simvastatin treatment in $CD44^{+}/CD24^{-}$ BCSCs

Uniprot ID	Protein names	Gene names	Ratio / Fold change a*
D3DPK5	SH3 domain binding glutamic acid-rich protein like 3	SH3BGRL3	1.5
B0QZ18	Copine-1	CPNE1	1.5
Q15084	Protein disulfide-isomerase A6	PDIA6	1.5
U6EFV3	MHC class I antigen (Fragment)	HLA-B	1.5
Q9NX34	cDNA FLJ20465 fis, clone KAT06236		1.5
G8JLB6	Heterogeneous nuclear ribonucleoprotein H	HNRNPH1	1.5
Q59FI9	Ribosomal protein L12 variant (Fragment)		1.5
P35579	Myosin-9	MYH9	1.5
Q59H77	T-complex protein 1 subunit gamma (Fragment)		1.5
P08758	Annexin A5	ANXA5	1.5
P62241	40S ribosomal protein S8	RPS8 OK/SW-cl.83	1.5
A0A087WVQ6	Clathrin heavy chain	CLTC hCG_1818599	1.5
Q92945	Far upstream element-binding protein 2	KHSRP FUBP2	1.5
P05387	60S acidic ribosomal protein P2 (Renal carcinoma antigen NY-REN-44)	RPLP2 D11S2243E RPP2	1.5
Q14847	LIM and SH3 domain protein 1 (LASP-1) (Metastatic lymph node gene 50 protein) (MLN 50)	LASP1 MLN50	1.5
Q15365	Poly(rC)-binding protein 1 (Alpha-CP1)	PCBP1	1.5
A8K4Z4	60S acidic ribosomal protein P0		1.5
H7BZJ3	Protein disulfide-isomerase A3 (Fragment)	PDIA3	1.6
A8K486	Peptidyl-prolyl cis-trans isomerase (PPIase)		1.6.
P06748	Nucleophosmin (NPM) (Nucleolar phosphoprotein B23) (Nucleolar protein NO38) (Numatrin)	NPM1 NPM	1.7
E9KL35	Epididymis tissue sperm binding protein Li 3a		1.7
A8K9C4	Elongation factor 1-alpha		1.7
P23396	40S ribosomal protein S3	RPS3 OK/SW-cl.26	1.7
P09211	Glutathione S-transferase P (GST class-pi) (GSTP1-1)	GSTP1 FAEES3 GST3	1.7
A0A0N9MPD2	MHC class I antigen (Fragment)	HLA-C	1.7
Q53YD7	EEF1G protein	EEF1G hCG_2039458	1.8
P15311	Ezrin (Cytovillin) (Villin-2) (p81)	EZR VIL2	1.8
P05023	Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	1.8
B7Z1K5	Tubulin alpha chain		1.8
V9HW80	Epididymis luminal protein 220 (Epididymis secretory protein Li 70)	HEL-S-70 HEL-220	1.9
H6VRG1	Keratin 1	KRT1	1.9
A4FRG0	MHC class I antigen	HLA-B	1.9
Q2TSD0	Glyceraldehyde-3-phosphate dehydrogenase		2.0
A0A0U1RRH7	Histone H2A		2.0
B2RDY9	Adenylyl cyclase-associated protein		2.0
P15531	Nucleoside diphosphate kinase A	NME1	2.1
A0A0U4BW16	Non-muscle myosin heavy chain 9	MYH9	2.1
Q05639	Elongation factor 1-alpha 2	EEF1A2	2.1
Q9NZ23	Drug-sensitive protein 1	YA61	2.3
E2QRB9	Thioredoxin reductase 1, cytoplasmic	TXNRD1	2.3
P62263	40S ribosomal protein S14	RPS14 PRO2640	2.4
P0C0S5	Histone H2A.Z (H2A/z)	H2AFZ H2AZ	2.4
J3KTL2	Serine/arginine-rich-splicing factor 1	SRSF1	2.5

P05783	Keratin, type I cytoskeletal 18	KRT18	2.6
P08727	Keratin, type I cytoskeletal 19	KRT19	2.6
P07900	Heat shock protein HSP 90-alpha (Heat shock 86 kDa)	HSP90AA1	2.7
P13639	Elongation factor 2 (EF-2)	EEF2 EF2	3.0
Q15366	Poly(rC)-binding protein 2	PCBP2	3.1

[a*] matched ID ratio (Fold changes) of differentially expressed proteins in CD44$^+$/CD24$^-$ BCSCs (Simvastatin – treatment versus control)

Table 2
Annotation of down-regulated proteins after Simvastatin treatment in CD44$^+$/CD24$^-$ BCSCs

Uniprot ID	Protein names	Gene names	Ratio / Fold change a*	
P12814	Alpha-actinin-1	ACTN1	0.5	
O75828	Carbonyl reductase [NADPH] 3	CBR3	0.5	
Q562Z4	Actin-like protein (Fragment)	ACT	0.5	
Q00839	Heterogeneous nuclear ribonucleoprotein U)	HNRNPU	0.5	
P04406	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	GAPDH GAPD	0.5	
P62736	Actin, aortic smooth muscle (Alpha-actin-2) (Cell growth-inhibiting gene 46 protein)	ACTA2 ACTSA ACTVS GIG46	0.5	
Q6FHK8	Phosphoglycerate mutase	PGAM1	0.5	
G9K388	YWHAE/FAM22A fusion protein (Fragment)	YWHAE/FAM22A fusion	0.4	
A0A142J2A5	MHC class I antigen	HLA-B	0.4	
P27348	14-3-3 protein theta (14-3-3 protein T-cell) (14-3-3 protein tau) (Protein HS1)	YWHAQ	0.4	
P31946	14-3-3 protein beta/alpha	YWHAB	0.4	
Q71U36	Tubulin alpha-1A chain (Alpha-tubulin 3)	TUBA1A TUBA3	0.4	
P68363	Tubulin alpha-1B chain (Alpha-tubulin ubiquitous)	TUBA1B	0.4	
Q9NZM1	Myoferlin (Fer-1-like protein 3)	MYOF FER1L3 KIAA1207	0.4	
A8K8D9	Glucose-6-phosphate 1-dehydrogenase		0.4	
Q6FG59	CDC37 protein	CDC37	0.4	
B4DJ30	cDNA FLJ61290, highly similar to Neutral alpha-glucosidase AB		0.4	
Q14974	Importin subunit beta-1 (Importin-90)	KPNB1 NTF97	0.4	
Q59EM9	Ubiquitin C variant (Fragment)		0.4	
P84098	60S ribosomal protein L19	RPL19	0.4	
P13667	Protein disulfide-isomerase (ERp72)	PDIA4	0.4	
P04792	Heat shock protein beta-1 (HspB1) (28 kDa heat shock protein)	HSPB1 HSP27 HSP28	0.4	
O43707	Alpha-actinin-4 (Non-muscle alpha-actinin 4)	ACTN4	0.4	
E9PK25	Cofilin-1	CFL1		0.4
Q14315	Filamin-C (FLN-C) (FLNc) (ABP-280-like protein) (ABP-L) (Actin-binding-like protein) (Filamin-2) (Gamma-filamin)	FLNC ABPL FLN2	0.3	
V9HW26	ATP synthase subunit alpha	HEL-S-123m hCG_23783	0.3	
P62805	Histone H4	HIST1H4A H4/A H4FA;	0.3	
P37802	Transgelin-2 (Epididymis tissue protein Li 7e) (SM22-alpha homolog)	TAGLN2 KIAA0120 CDABP0035	0.3	
V9HW31	ATP synthase subunit beta (EC 3.6.3.14)	HEL-S-271	0.3	
P05787	Keratin, type II cytoskeletal 8 (Cytokeratin-8) (CK-8) (Keratin-8) (K8) (Type-II keratin Kb8)	KRT8 CYK8	0.3	
Q0D2Q6	Phosphoglycerate mutase	PGAM1		0.3
P07437	Tubulin beta chain (Tubulin beta-5 chain)	TUBB TUBB5 OK/SW-cl.56	0.3	
V9HWG3	Epididymis secretory protein Li 45	HEL-S-45	0.3	
P50454	Serpin H1 (47 kDa heat shock protein	SERPINH1	0.3	
P00338	L-lactate dehydrogenase A chain (LDH-A) carcinoma antigen NY-REN-59)	LDHA	0.3	
P18206	Vinculin (Metavinculin) (MV)	VCL	0.3	
Q8IWP6	Tubulin beta chain		0.3	

P08670	Vimentin	VIM	0.3
P60709	Actin, cytoplasmic 1 (Beta-actin) [Cleaved into: Actin, cytoplasmic 1, N-terminally processed]	ACTB	0.3
Q59EJ3	Heat shock 70kDa protein 1A variant (Fragment)		0.3
Q9Y490	Talin-1	TLN1 KIAA1027 TLN	0.3
P14625	Endoplasmin (94 kDa glucose-regulated protein) (GRP-94) (Heat shock protein 90 kDa beta member 1) (Tumor rejection antigen 1) (gp96 homolog)	HSP90B1 GRP94 TRA1	0.3
P07355	Annexin A2	ANXA2	0.3
J3KPS3	Fructose-bisphosphate aldolase (EC 4.1.2.13)	ALDOA hCG_1811258	0.3
P08729	Keratin, type II cytoskeletal 7	KRT7	0.2
P06733	Alpha-enolase	ENO1	0.2
Q15149	Plectin (PCN) (PLTN) (Hemidesmosomal protein 1) (HD1) (Plectin-1)	PLEC PLEC1	0.2
P21333	Filamin-A (FLN-A) (Actin-binding protein 280) (ABP-280) (Alpha-filamin) (Endothelial actin-binding protein) (Filamin-1) (Non-muscle filamin)	FLNA FLN FLN1	0.2
P22626	Heterogeneous nuclear ribonucleoproteins A2/B1 (hnRNP A2/B1)	HNRNPA2B1 HNRPA2B1	0.2
A0A024R321	Filamin B, beta (Actin binding protein 278), isoform CRA_a	FLNB hCG_27732	0.2
O60664	Perilipin-3	PLIN3	0.2
P10809	60 kDa heat shock protein, mitochondrial (60 kDa chaperonin)	HSPD1 HSP60	0.2

[a*] ***matched ID ratio (Fold changes) of differentially expressed proteins in CD44^{+}/CD24^{-} BCSCs (Simvastatin – treatment versus control***

Table 3.

KEGG Pathway enrichment analysis of 93 Differentially expressed proteins in BCSCs treated with Simvastatin $p < 0.05$

#pathway ID	pathway description	observed gene count	false discovery rate
3010	Ribosome	7	0.000108
4510	Focal adhesion	7	0.00134
4810	Regulation of actin cytoskeleton	7	0.00134
4141	Protein processing in endoplasmic reticulum	6	0.00256
4670	Leukocyte transendothelial migration	5	0.00413
4520	Adherens junction	4	0.00703
4145	Phagosome	5	0.00904
4540	Gap junction	4	0.00949
1200	Carbon metabolism	4	0.0171
5203	Viral carcinogenesis	5	0.0171
4530	Tight junction	4	0.0292
10	Glycolysis / Gluconeogenesis	3	0.0294
4151	PI3K-Akt signaling pathway	6	0.0346
1230	Biosynthesis of amino acids	3	0.0353
4390	Hippo signaling pathway	4	0.0353
4918	Thyroid hormone synthesis	3	0.0353
4971	Gastric acid secretion	3	0.0353
5412	Arrhythmogenic right ventricular cardiomyopathy (ARVC)	3	0.038

DISCUSSION

Simvastatin, a potent HMG CoA reductase inhibitor is a well established lipid lowering drug in clinical settings. Recently, it's gaining significant importance in targeting cancer stem cells. It has been shown that, Simvastatin reduces the expression of CSC surface markers and sphere forming ability in cancer stem cells. However their molecular mechanism remains poorly understood.$CD44^{+}$ / $CD24^{-}$ BCSCs sorted form MDA-MB-231 cell line was used in this study . 48 hrs treatment of BCSCs with various concentrations of Simvastatin showed a remarkable anti-proliferative activity with the IC50 value of 5 µg/ml . Guava Nexin staining of the treated cells clearly indicates that, Simvastatin induces apoptosis in BCSCs .Furthermore, Mammosphere formation was significantly inhibited upon Simvastatin treatment. Collectively, these results suggested that Simvastatin has the inherent ability to inhibit BCSCs growth, induce apoptosis and inhibit mammosphere formation invitro.Proteomics, specifically Label free quantitative proteomic profiling, is a unique method to investigate the drug action at its functional level. In this profiling of Simvastatin treated and untreated BCSCs, 99 differentially expressed proteins were identified of which, 48 were up regulated and 51 were down regulated. Gene ontology and KEGG pathway enrichment analysis exposed 18 potential pathways associated with Simvastatin treatment (Table 3). These identified pathways were shown to be related with carcinogenesis, tumor progression, metastasis, focal adhesion and metabolic effects in cancer cells. Among the down regulated proteins Vinculin, Heat shock protein beta-1(HSPB1) was significantly down regulated.

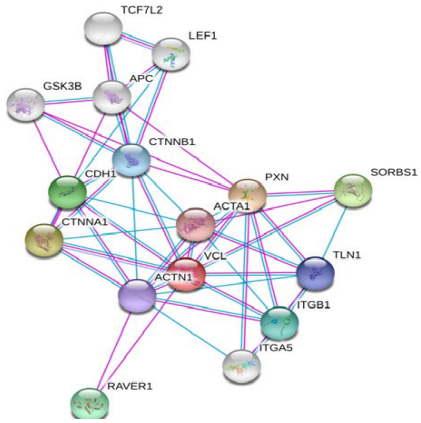
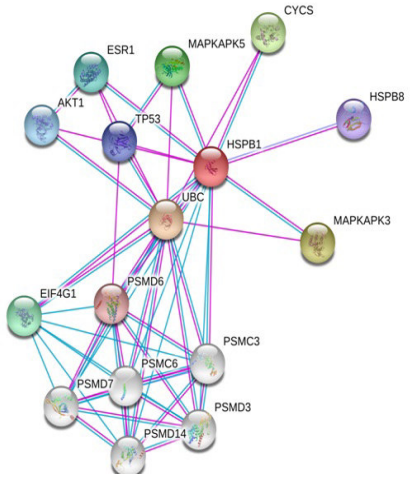


Figure 7
Vinculin interactome network



Network built by using string vs 10 is shown. Different line colors represent the types of evidence of the association: pink for experimental and blue for databases

Figure 8
HSPB1 interactome network

Further, Vinculin protein interactome analysis was performed, which revealed that vinculin rewires multiple proteins associated with focal adhesion, Wnt signaling pathway, pathways in basal cell carcinoma , proteoglycon in cancer and insulin signaling pathways (Figure 7). Studies in human mesenchymal stem cells show that vinculin acts as a mechanosensor and regulates stem cell behavior and mesenchymal differentiation. Further it has been linked with extracellular stiffness[12,13]. Collectively these findings suggest that, the adherent protein vinculin can regulate the longevity of cancer stem cells.HSPB1 protein,on interactome analysis clearly exhibits its association with apoptosis, mutiple oncogeneic pathways, MAPK signaling pathway, VEGF signaling pathways and p53 signaling pathways (Figure 8).Many studies have shown that HSPB1 over expression in breast carcinoma affects the sensitivity of cancer to chemotherapy,radiotherapy and the final outcome.[14,15] Further it has been shown that HSPB1 regulates EMT.[16] Thus HSPB1 can be proposed to be a potential target in BCSCs . These results demonstrated the potential use of Simvastatin in cancer therapeutics. Other proteins involved in signaling pathways related to CSCs and cancer which were significantly down regulated by Simvastatin include, Fructose-bisphasphate aldolase, Phosphoglycerate mutase , Talin-1 and cofilin-1.Fructose-bisphosphate aldolase A (ALDOA) is a key enzyme in glycolysis and is responsible for catalyzing the reversible conversion of fructose-1,6-bisphosphate to glyceraldehydes-3-phosphate and dihydroxyacetone phosphate. ALDOA contributes to various cellular functions such as muscle maintenance, regulation of cell shape and mobility, striated muscle contraction, actin filament organization and ATP biosynthetic process.[17] Here, we report that ALDOA is down regulated upon Simvastatin treatment. Further, its expression level is correlated with tumor metastasis, grades, differentiation status and favorable prognosis[17]. These data suggest that ALDOA could be a potential marker and therapeutic target for drug development.Phosphoglycerate mutase 1 (PGAM1) catalyzes the conversion of 3-phosphoglycerate (3-PG) to 2-phosphoglycerate (2-PG) in glycolysis process. It has been shown that, it regulates a distinctive step in glycolysis, where most of the glycolytic intermediates are used as precursors for anabolic biosynthesis.[18] Furthermore, PGAM1 gene expression is up regulated in many cancer cells due to loss of TP53.[19] Treatment with Simvastatin has been found to result in significant downregulation of this protein, making it a vulnerable target for new drug development.Talin-1, a cytoskeletal protein has been shown to be associated with neoplastic adhesion, invasion and migration of cancer cells.[20] Its expression has been shown to be high in triple negative breast cancer cells.[21,22] Further low expression is significantly correlated with better prognosis of cancer treatment.[22,23] Interestingly, in our study Simvastatin significantly reduces the expression of Tailn-1. Suggesting, Talin-1 as a potential target in cancer therapeutics.Recent evidence suggests that, the cofilin pathway is significantly associated with tumor metastasis. Studies indicate that the overall activity of the cofilin pathway, determines the invasive and metastatic phenotype of tumor cells.[24,25] Further in our study Cofilin-1 is significantly down regulated by Simvastatin, suggesting the potential role of Simvastatin in therapeutic benefit in combating tumor metastasis.Overall, our study reveals that Simvastatin potentially targets BCSCs and regulates multiple signaling pathways associated with CSCs and cancer cells such as cancer metastasis, progression, apoptosis, EMT and focal adhesion.

CONCLUSION

Our finding implies that the use of Simvastatin is a promising new method to control breast cancer stem cells. Further our proteomics profiling implies the better use of existing drugs and new targets in drug development

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CONFLICTS OF INTEREST

Conflict of interest declared none.

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