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Therapeutic Potentials of CD151 shRNA in Targeting Metastasis of Triple Negative Breast Cancer Cell Line MDA-MB-231

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Abstract

Objective: CD151 is a master regulator of cell adhesion signalling and acts as a promoter in tumour progression. Induction of RNAi through shRNA expression holds great prospective in biomedical research. Bioinformatics approach used to predict potential shRNAs that knockdown CD151. The aim of present study is to investigate the role of CD151 in metastasis of triple negative breast cancer cells using shRNA.

Methods: Triple negative breast cancer line, MDA-MB-231 obtained from NCCS Pune, India and expression of CD151 determined using western blot and RT-PCR analysis. Small hairpin RNA (shRNA) was constructed using pSilencer 2.1-U6 puro vector to knockdown CD151 expression. The role of CD151 in proliferation, apoptosis, migration, invasion and cell adhesion was evaluated by silencing CD151 gene using CD151shRNA.

Results: RNA interference technology (RNAi) used to silence CD151 gene expression in MDA-MB-231 cells. Delivery of specific shRNA targeting human endogenous CD151 showed significant growth inhibition of MDA-MB-231 cells. The gene expression study by RT-PCR analysis showed that expression of CD151 at mRNA level reduced six fold with CD151 gene knockdown. CD151 gene silencing for 48h using shRNA decreased proliferation by 62.7%. CD151 knockdown also lead to the significant inhibition of metastasis and induced apoptosis.

Conclusion: CD151 over expression is essential for tumour progression and our study shows that shRNA mediated gene silencing of CD151 decreases the metastasis, thus emphasizing CD151 as a prognostic marker and help in developing new therapeutics for treatment of triple negative breast cancer.

Keywords: CD151; shRNA; MDA-MB-231; Metastasis; Apoptosis

Introduction

Cancer is well recognized as a global health problem for decades. It defined as collection of diseases characterized by abnormal cell growth, violating the cell division rules and spreads to the surrounding tissues. While specific signals dictate the cell division in healthy cells, cancer cells develop a degree of autonomy resulting in uncontrolled proliferation leading to tumour development. Most of the cancer-related deaths (90%) are due to spreading of tumour cells to other body parts, a process called as metastasis.

Breast cancer is one of the most common cancers diagnosed in women in India and worldwide. It is the second most frequent cause of death due to cancer in women. In the year 2015, 1,55,000 new breast cancer cases were expected and of, 76,000 women were expected to die. WHO ironically stated that most of the breast cancer cases in India were diagnosing at stage III or IV that drastically affects the survival rate and treatment options. Today there are choices of treatment options that fight with complex mix of cells in breast cancer, including surgery, radiation, hormonal (anti-estrogen) therapy and chemotherapy [1]. Majority of breast cancer deaths are because of ineffective treatment of metastatic disease. Therefore, metastatic breast cancer patients need to treat with targeted therapies directed at specific molecular alterations.

The tetraspanin transmembrane proteins have emerged as key gamer in malignancy and have variety of specific molecular interactions through formation of tetraspanin-enriched microdomains [2]. Tetraspanins have emerged as diagnostic and prognostic markers and gained increased attention due to their functional versatility [3]. CD151 is one among them, reported as an adaptor molecule [4]. It is expressed on normal cells but over expressed on cancer cells. It functions at multiple cancer stages [5], particularly implicated in tumour metastasis [6]. It also functions as an important regulator of communication between tumour cells and endothelial cells [7], carcinoma-stroma interactions cell-cell and cell-ECM interactions [8]. Earlier, the role of CD151 demonstrated in variety of signalling mechanisms [9-14]. It promotes tumour angiogenesis [15], growth and survival [16] and metastasis [17-20]. High expression of CD151 confers poor prognosis in breast [21]. Our recent review highlighted the role of CD151 in tumour metastasis and its importance in cancer therapy [22]. CD151 may represent new therapeutic target as it is simultaneously affecting the proteolytic activity of different enzymes, signalling and different cellular mechanisms.

Targeting of CD151 using monoclonal antibodies displayed anti-metastatic activity *in vivo* [5]. Blocking of CD151 using mAbs inhibited the tumour growth [5], migration [23] and metastasis [18] in several cancers. Several limitations highlighted in clinical and animal studies hampered the use of mAbs in cancer therapy. In addition, the large size of mAbs decreases the penetration and retention of the therapeutic antibody in the malignant tissues and thus reducing the efficiency of the treatment [24].

RNA interference (RNAi) is relatively innovative discovery for inhibiting gene expression and target mediated treatment of cancer.

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Earlier methodologies have incorporated antisense technology and catalytic ribozymes. However, the efficacy of these approaches is mixed, particularly in inhibiting established tumours. The stableness/specificity of the oligonucleotides and issues in target collection choice impede the extensive use of those techniques [25]. Analysis of the kinetics of siRNA-mediated gene silencing reveals that the gene inhibition by unmodified siRNAs can last for a week in rapidly dividing cells and up to one month in cells with minimal division. shRNA is highly effective tool used to silence expression of specific genes. RNA interference (RNAi) mediated by small interfering RNAs (siRNAs) or short hairpin RNAs (shRNAs) has emerged as a powerful genetic tool to investigate the functional studies. There are several methods to induce RNAi for gene knockdown [26] but commonly used methods are chemically synthesized siRNAs and shRNA expression vectors. Compared to chemically synthesized siRNAs, vector-based shRNA induce transient expression when introduced in to the vector [27].

Targeting of CD151 using shRNA can overcome the limitations of using mAbs, as shRNA are highly specific and smaller. Moreover, synthesis of CD151 shRNA is inexpensive as it is only a 20-25 nt long RNA fragment. shRNA is chemically modified and introduced in to cells by using expression vectors. This type of expression plasmids transiently expressed in the cell and show stable gene silencing.

Materials and Methods

Prediction of cellular, molecular and biological functions of CD151

CD151 protein network in Homo sapiens constructed using STRING online search tool, subjected to K-mean clustering and its role in biological process, molecular functions and cellular components analyzed by enrichment with genome, kinome and druggable backgrounds.

Prediction of CD151 gene expression pattern using GENT database

CD151 expression pattern in human normal and tumour tissues, subtypes and cancer cell line retrieved from GENT database created by the Afymetrix U133Plus2 and U133A platforms (http://medicalgenome.kribb.re.kr/GENT/ or genome.kobic.re.kr/GENT/). Expression of CD151 detected by cancer outlier profile analysis (COPA) and visualized by box-plot [28].

Design of CD151 shRNA template oligonucleotides

Gene silencing using shRNA is a rapidly evolving tool in molecular biology. By scanning CD151 mRNA (cds) for AA dinucleotide sequences start from AUG and potential target sequences from +91, +210, +393 and +824 were identified [29]. Finally two sets of potential target sequences were obtained by comparing above target sequences to human genome database and by eliminating target sequences that have homology of 16-17 contiguous base pairs to other coding sequences using BLAST search (5'-GATCCGAGTTCAACGAGA-AGAAGACATCAAGAGTGTCTTCTTCTCGTTGAACTCTTTTTG-GAAA-3' and 3'-GCTCAAGTTG CTCTTCTTCTGTAGTTC TCACAGAAGAAGAGCAAC TTGAGAAAAACC TTT TCGA-5′ for construct A (pCD-A); 5'- GATCCGGAGATCATCGCTGG-TATCCTTCAAGAGAGG AT AC C AGCGATGATCTCCTTTTTG-GAAA-3′ and 3'GCCTCTAGTAGCGACCATAGGA AGTTC TCTC CTAT GGTCGCTACTAGAGGAAAAACCTTTTCGA-5′ for construct B (pCD-B). Hairpin siRNA template oligonucleotides were designed from target sequences using web-based insert design tool (www.ambion.com/techlib/misc/ pSilencer_converter.html).

Construction of CD151shRNA expressing vector

Desalted complementary oligonucleotides with loop sequence of 5'-UUCAAGAGA-3' at centre and 5 nucleotide poly (T) tract as a termination signal for RNA pol III at 3'-end and *Bam*H1 or *Hind*III restriction site at 5'-end was commercially obtained. Two self-complementary oligonucleotides annealed by heating at 90°C for 3min and cooled to room temperature in 6x SSC which leads to formation of dsDNA molecules with respective sticky restriction site ends. These dsDNA molecules were ligated to linearized p*Silencer* 2.1-u6 puro vector between *Bam*H1 and *Hind*III restriction sites. This finally resulted in a shRNA expression plasmid for CD151 (pCD). Non-targeting negative control shRNA (pSV) also constructed with scrambled nucleotide sequence of the CD151 shRNA.

Identification of clones with shRNA inserts

Standard protocol used for transformation of *E. coli* cells [30]. CD151 shRNA expression plasmid (5 µl) added to 100 µl of DH5α competent cells. The content was kept in water bath at 42°C for 90 sec for heat shock treatment and immediately chilled for 5min. 1ml of LB broth was added and incubated at 37°C for 45 min. Cell suspension (100 µl) from each transformed culture was added to LB agar plate containing ampicillin (100 µg/ml) and incubated overnight at 37°C. Isolated colonies were selected and inoculated in to 4 ml of LB broth containing ampicillin (100 µg/ml) and incubated at 37°C for 8 h with vigorous shaking (200 rpm). 100 µl of this starter culture was added to 100 ml of LB medium containing ampicillin (100 µg/ml) and grown overnight at 37°C with vigorous shaking (200 rpm). Plasmid was isolated and purified using QIAGEN Plasmid Maxi kits as per the manufacture's protocol. The isolated plasmid digested with BamH1 and Hind III to confirm presence of the shRNA template insert.

Cell culture and transfection

The MDA-MB-231 is triple negative breast cancer cell line, established from human pleural effusions, obtained from NCCS, Pune, India. Cells were grown in Leibovitz's L-15 medium supplemented with 10% fetal bovine serum in a free gas exchange with atmospheric air at 37°C. MCF-12A, a non-tumourigenic breast epithelial cell line, was grown in a mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium (1:1) containing 20 ng/ml human epidermal growth factor, 100 ng/ml cholera toxin, 0.01 mg/ml bovine insulin and 500 ng/ml hydrocortisone and 5% horse serum under humidified atmosphere of 5% CO_2. Monolayer of MDA-MB-231 cells grown overnight (~80% confluence) were transfected with 1-2 µg of pSV or pCD shRNA plasmid using Lipofectamine® 3000 Reagent (Life Technologies, USA) as per manufacturer's instructions. After 8 h of transfection, the medium replaced with serum containing medium and incubated for 24 or 48 h. Expression of CD151 determined by RT-PCR analysis and compared with control (pSV).

Reverse transcription-PCR analysis of down regulation of CD151 mRNA with pCD151

Total RNA was extracted using TRIZOL reagent as per manufacturer's instructions (Invitrogen.USA). DNase-treated RNA used as a template for reverse transcription reaction followed by PCR analysis (Qiagen, USA) using sequence specific primers for CD151 and GAPDH (Table 1). The PCR conditions were as follows: 3 min at 93°C for initial denaturation of template DNA, 35cycles at 93°C for 15sec-denaturation, annealing at 62°C for 30sec and extension at 68°C for 1 min/Kb, with a final extension step of 7 min at 72°C. The PCR products were analysed on in 1% agarose gel electrophoresis. Intensity of bands was measured using Image J software (NIH, USA).

Gene	Forward primer	Reverse primer
CD151	5′-CAAGGCCGAGATCGTTCC-3′	5′-ACAGCAGTGTACAAAACCAGG-3′
GAPDH	5'-CAAGGTCATCCATGACAACTTTG-3'	5'-GTCCACCACCCTGTTGCTGTAG-3'

Table 1: Genes analysed by RT-PCR.

MTT assay

MTT assay used to evaluate the effect of CD151 gene silencing using pCD on proliferation of MDA-MB-231 and MCF-12A cells. Monolayer cells in 96-well plate (5×10^3/well) were transfected with 1 µg of pCD-B for 24 and 48 h using Lipofectamine. pSV transfected cells maintained under similar conditions to serve as the control. After transfection, MTT reagent (5 mg/ml of PBS) added to each well, incubated for 2 h for color development and DMSO was added to solubilize formazan crystals. Absorbance at 595 nm was measured using ELISA plate reader. The percent reduction of cell viability expressed by comparing with to pSV treated cells, which set as 100%.

Brdu incorporation assay

BrdU incorporation assay used to measure effect of pCD on cell proliferation. Cells (10×10^3/well) were seeded in triplicate into 96-well plates and allowed to grow overnight before transfection with pSV or pCD for 48 h. After incubation, cells were labeled with diluted BrdU (1:2000 in tissue culture medium) for12 h. Cells were fixed with fixative solution and incubated for 30 min at room temperature. After incubation, anti-BrdU antibody (1:100) added and incubated for 1 h at room temperature. After washing, conjugate antibody (1:2000) added and incubated for 30 min at room temperature. After washing, tetra-methylbenzidine substrate added and incubated in dark for 15 min. Then stop solution was added and absorbance was read at dual wavelength of 450 and 540 nm within 30 min.

Adhesion assay

The effect of CD151 gene silencing using pCD on the adhesive properties of MDA-MB- 231 breast cancer cells was determined by Adhesion assay. 96-well plate was coated with various ECM components. Briefly, 100 µL of fibronectin (10 µg/mL; Sigma-Aldrich), vitronectin (5 µg/mL; Invitrogen), laminin (5 µg/mL; Sigma-Aldrich) and collagen I (50 µg/mL; Invitrogen) were added separately to each well and incubated at 37°C for 1 hr. The wells were blocked with 0.2% BSA for 2 h at room temperature. After washing with PBS, 100 µl of suspension of cells transfected earlier with pSV and pCD151 for 48 h were loaded in to each well. The cells were allowed to adhere for 2 h, then washed carefully, fixed with methanol and stained with Hema-3 stain. The cells were extracted into 10% methanol and 5% acetic acid and absorbance was read at 650 nm using ELISA plate reader.

Wound scratch assay

To study cell migration, cells were seeded in a 6-well plate at density of (2×10^6/well) and transfected with pSV or pCD for 48 h. Then, a straight scratch was made in individual wells with a 200 µL pipette tip. This point was considered the "0 h," and the width of the wound was photographed under the microscope. Then wound healing was checked under microscope and photographed at 0 h, 24 h and 48 h. Wound healing was measured by calculating the reduction in the width of the wound after incubation and compared to 0h which is set as 100%.

Matrigel invasion assay

Matrigel assay used to determine the effect of pCD on invasive ability of MDA-MB-231 cells. Breast cancer cells (2×10^5/well) were transfected with pSV and pCD for 48 h. After the incubation period, cells were trypsinized and suspended in 100 µL of serum-free medium. The cells were seeded into the upper chamber of Boyden chamber filters (8 µm pore) coated with Matrigel (50 µg/filter) and 600 µL complete medium was added to the lower chamber. The cell were allowed to migrate for 12 h. The cells invaded through the Matrigel coated inserts were fixed with formaldehyde (3.7% in PBS), stained with 0.4% crystal violet, and destained twice with PBS. Non invaded cells were scrapped with cotton swab and counted in random microscopic fields.

Tunnel assay

One of the most widely adopted method of choice for detecting DNA damage *in situ* is TdT-mediated dUTP-biotin nick end labelling (TUNEL) assay. Cells were transfected with pSV and pCD for 48 and 72 h. After transfection, cells fixed with 4% Para-formaldehyde and incubated with a reaction mixture containing biotin-dUTP and terminal deoxynucleotidyl transferase for 1h. Then cells stained with DAPI and fluorescein-labelled cells visualized with fluorescent microscopy and expressed as percent compared to DAPI stained cells.

Statistical analysis

Statistical analysis and graphical exploration of the data were done using Microsoft Excel. All the experiments were carried out three times. Values are shown as means ±SD of at least three independent experiments. Significance set as $p<0.001$.

Results

Functional analysis of CD151

Understating the role of CD151 is required to target survival, angiogenesis and metastasis of cancers. search tool for the retrieval of interacting genes/proteins (STRING) is a biological database developed by a consortium of academic institutions. To predict gene ontology, CD151 interaction partners were clustered using k-means and enrichment of the associated functional annotations measured to generate putative functions. These results show that CD151 is a key cellular component of membrane, focal adhesion, extracellular matrix, basement membrane and cell junctions. CD151 was predicted to be actively involved in biological processes like extracellular matrix organization, hemidesmosome assembly, cellular component organization and cell junction assembly. During which it may perform several molecular functions such as receptor binding, ligase activity, ubiquitin like protein transferase activity and adhesion molecular binding. Further, human disease associations (http://tissues.jensenlab.org) and tissue annotations (http://diseases.jensenlab.org) of CD151 were done using enrichment detection. The results revealed that CD151 associate with cancer having Z-score of 3.6 and confidence of 2.0. The results from tissue annotations indicate that CD151 protein localized mostly in breast glandular cells and low in myoepithelial cells but not detected in adipose tissue.

CD151 gene expression pattern in human normal and tumour tissues, subtypes and cancer cell lines

The use of focused treatments directed at specific molecular change benefits breast cancer sufferers. Human tissue gene expression facts sets

produced the usage of Affymetrix U133A and U133lPus2 platforms constructed from public sources and huge-scale gene expression database of greater than 40,000 samples used to explore for CD151 over expression. The result shows that CD151 was over expressed in subset of breast, kidney, lung and brain tumour tissues (Figures 1a and 1b) and in subset of cell lines from those four tissues (Figures 1c and 1d).

Endogenous expression of CD151 in MDA-MB-231 and MCF-12A

Triple negative breast cancer (TNBC) represents about 10-15% of all breast cancers and sufferers with TNBC have a poor prognosis compared to the other subtypes [31]. Given the lack of proven molecular targets and the poor final results in sufferers with TNBC, there's a transparent need for identification of molecular target. Most cancer cell lines have proved beneficial in laboratory and preclinical investigations [32]. Review of the literature found twenty- seven human breast cancer cell lines, which classified as TNBC cell lines. The MDA-MB-231 breast cancer cell line obtained from a patient in 1973 at M. D. Anderson Cancer Centre. MDA-MB-231 breast cancer cells appear in spindle shape with epithelial-like morphology and invasive phenotype [33].

Preliminary study using GENT database predicted over expression of CD151 in MDA-MB-231 breast cancer cell line (Figures 2a and 2b). Hence, MDA-MB-231 cells used to evaluate the effect of CD151 shRNA on metastasis. We first attempted to compare the expression level of CD151 in human triple negative breast cancer cell line, MDA-MB-231 with human normal breast epithelial cell line, MCF-12A using western blot and RT PCR analysis. As shown in Figure 2c, CD151 protein level was significantly higher in MDA-MB-231cells as compared with the MCF-12A, which expressed undetectable levels of this protein. Similar trend seen in CD151 expression at mRNA level as assessed by RT-PCR analysis (Figure 2d).

Knockdown of CD151 gene expression in MDA-MB-231 triple negative breast cancer cells using RNAi

To study the biological role of CD151 in triple negative breast cancer metastasis, we used small hairpin RNAs to knockdown endogenous CD151 gene expression in MDA-MB-231 cells, which showed robust expression of CD151 as well as high metastatic potential. First, we constructed two sets of *pSilencer* vectors containing small hairpin capable of generating 21-nucleotide duplex RNAi oligonucleotides corresponding to CD151 (Figure 3a). Presence of shRNA insert confirmed by restriction digestion followed by electrophoresis (Data not shown).Then vectors expressing shRNA-for CD151 were transfected into MDA-MB-231 cells for 24 and 48 h. Analysis of transfected cells via semi-quantitative reverse transcription-PCR demonstrated significant reduction in CD151 expression at mRNA level with pCD-B (Figure 3b). Densitometric analysis using Image J software indicates the reduction of CD151 expression at mRNA by 63.8% at 24 h and 85% at 48 h relative to pSV-transfected cells, which set as 100% (Figure 3c). However, effect of pCD-A plasmid on expression of CD151 was negligible (Data not shown). Conversely, no effect of pCD was observed on the expression of GAPDH, which used as an internal control for specificity and loading at mRNA level. As pSV transfection did not effected mRNA expression indicating that RNAi-directed CD151 knockdown is specific.

Knockdown of CD151 expression by RNAi induces toxicity in triple negative breast cancer

As preliminary study, cytotoxic effect of CD151 shRNA expression plasmids on non-tumourigenic cell line, MCF-12A was tested. After 48 h of transfection with 1 µg of shRNA specific to CD151, viability of MCF-12A cells was ~98% (Figure 4a). To test the potential cytotoxic effects of RNAi-mediated CD151 silencing on viability of MDA-MB-231 cells, MTT assay performed after transfection with 1 µg of pCD for 24 and 48 h. The results showed that there is a significant reduction of viability with shRNA targeting CD151. Percent of viable cells was 46.2

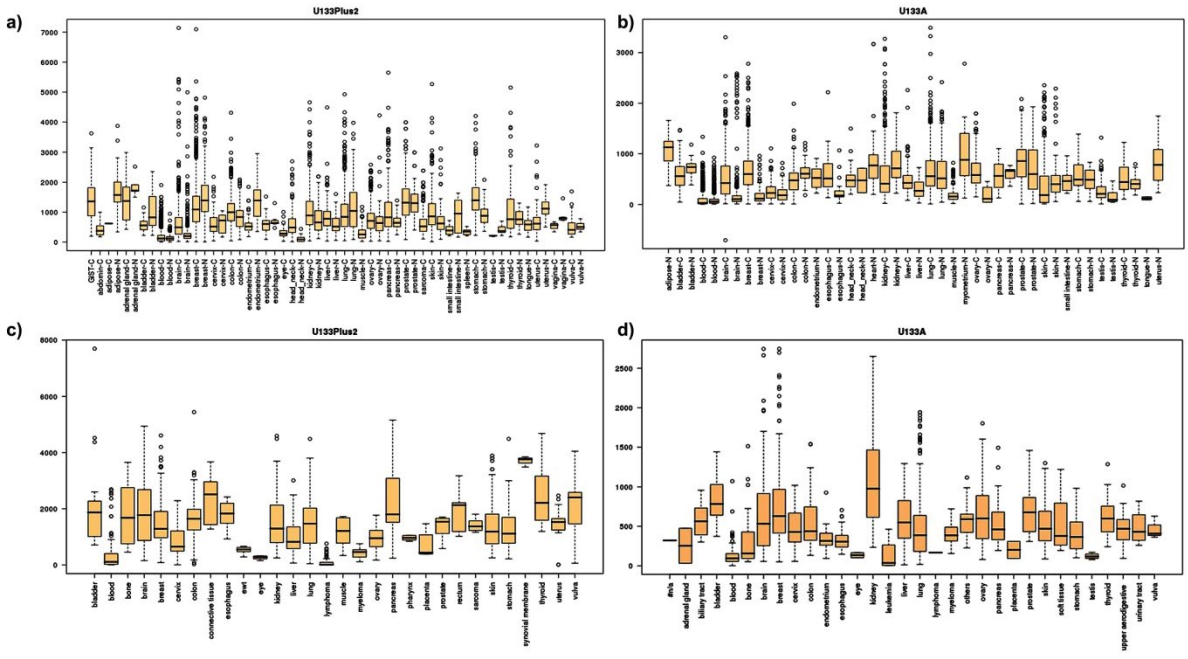

Figure 1: Pattern of CD151 expression across diverse normal tissues, tumour tissues and cell lines. U133plus2 data set (a) and U133A data set (b) of expression pattern of CD151 across diverse normal and tumour tissues. Expression pattern of CD151 in diverse cell lines (c and d).

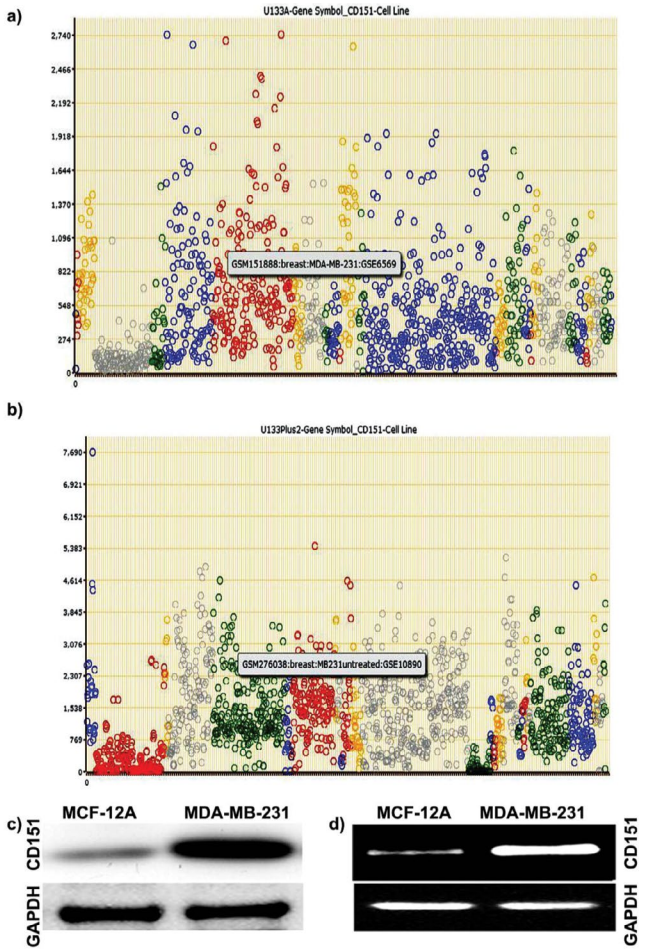

Figure 2: Expression of CD151 in human triple negative breast cancer cell line MDA-MB 231. Expression pattern of CD151 in MDA-MB-231 triple negative breast cancer cell line. a) U133A data and (b) U133plus2 data set of flash chart. Expression of CD151 in MDA-MB-231 breast cancer cells and MCF-12A normal breast epithelial cells as determined by Western blot (c) and RT-PCR analysis (d).

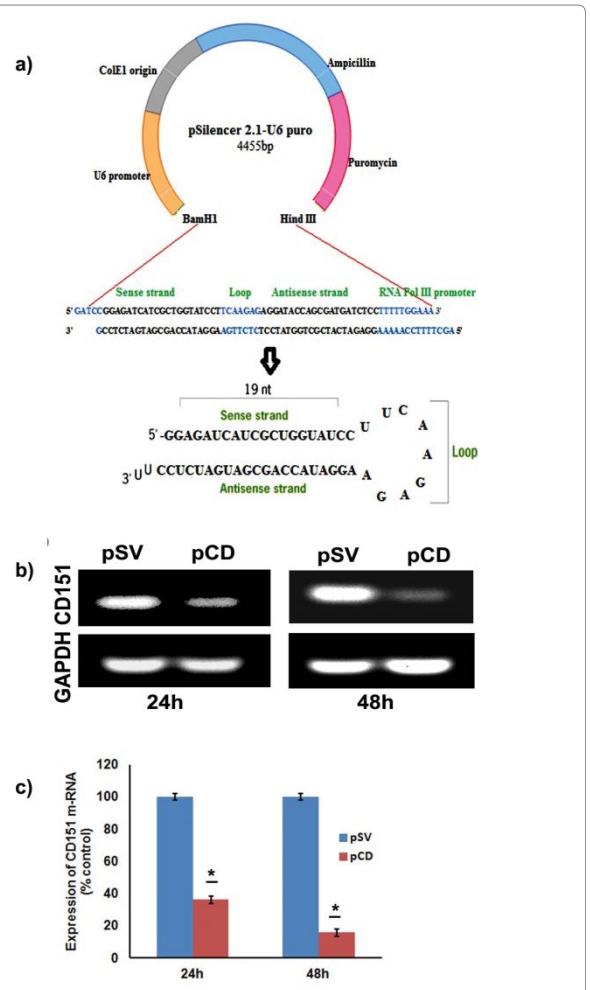

Figure 3: Targetting CD151 in MDA-MB-231 cells using shRNA construct a) Schematic representation of shRNA expressing plasmid construction from *pSilencer* 2.1-u6 puro vector. b) Total RNA was isolated from pSV and pCD treated cells and expression of CD151 at mRNA level was determined by RT-PCR using specific primers. c) Quantitative analysis of CD151 mRNA expression by Image J software. The mean density of CD151 within the management cluster was outlined as100%. Data are presented as means ±SEM (n=3, **p*<0.001).

and 45.4% with shRNA-A; 41.7 and 37.3% with shRNA-B at 24 and 48 h, respectively compared to pSV control indicating time dependent reduction of viability specific to CD151 gene silencing (Figure 4b). These outcomes imply that pCD-B (shRNA-B) has potential in silencing CD151 gene expression and cytotoxicity compared to pCD-A. Hence, pCD-B used for further studies and represented as pCD.

Knockdown of CD151 expression by RNAi inhibits proliferation

BrdU incorporation assay performed to analyze the effect of CD151down regulation on proliferation. The results showed that the knockdown of CD151 using CD151 shRNA resulted in a significant reduction of proliferation by 36 and 65% at 24 and 48 h, respectively (Figure 4c) compared to pSV transfected cells, which set as 100%.

Knockdown of CD151 expression by RNAi induces apoptosis

To investigate whether RNAi mediated CD151 down regulation induce apoptosis, MDA-MB-231 cells were transiently transfected with the pSV and pCD for 48 and 72 h and apoptosis was analysed by Tunnel assay (Figures 4d and 4e). The results show that apoptotic rate of pCD transfected cells was approximately 54 and 63%, respectively at 48 and 72 h compared to DAPI stained cells (Figure 4f). However, apoptotic rate of pSV transfected cells was negligible. The results indicate that down regulation of CD151 expression by RNAi triggers apoptosis in MDA-MB- 231 cells.

Knockdown of CD151 expression by RNAi inhibits cell migration and invasive potential of MDA-MB-231 cells

The effect of CD151 gene silencing on migration of MDA-MB-231cells was determined by wound scratch assay. The wound healing ability was observed after transfection with pSV and pCD at 0, 24 and 48 h. In pSV transfected cells, the area of wound was significantly decreased compared to pCD treated cells (Figure 5a). The wound area at 0 h was set as 100% in both pSV and pCD treated cells. The wound area at 24 and 48 h was 60 and 2%, respectively in pSV treated cells, whereas 80 and 60%, respectively in pCD treated cells compared to wound area at 0 h. These results indicate that percent of wound repair in pCD treated cells decreased by 20% at 24 h and 58% at 48 h

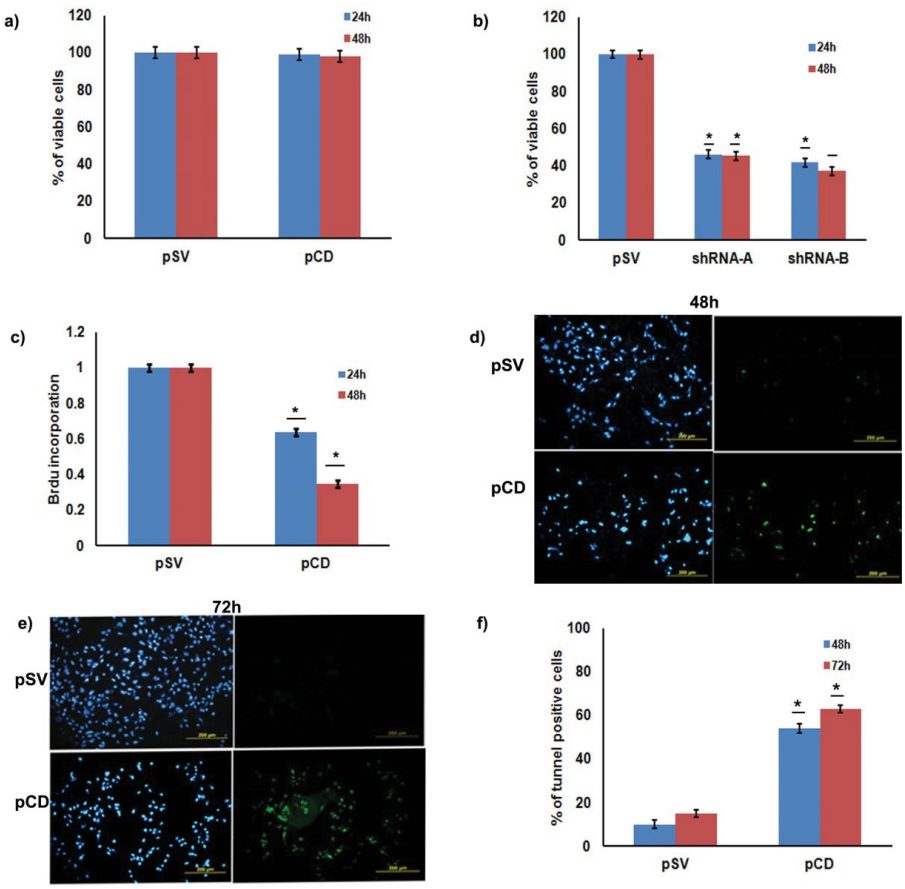

Figure 4: Effect of CD151 gene silencing on proliferation and apoptosis of MDA-MB-231 cells. Effect of pCD on viability of MCF-12A (a) and MDA-MB-231 (b) cells at 24 and 48 h. Results were expressed in percent control. c) Effect of pCD on BrdU incorporation during proliferation of MDA-MB-231 cells. The results were expressed in percent control. Effect of pCD on induction of apoptosis in MDA-MB-231 cells at 48 h (d) 72 h (e). Tunnel positive cells were counted using Image J (NIH, USA) and expressed as percent of DAPI stained cells (f). The cumulative data of each assay was done from 3 independent experiments and shown as means ±SEM (n=3, *p<0.001).

compared to pSV treated cells (Figure 5b). The most precarious nature of malignancy is metastatic potential. The down regulation of CD151 using pCD reduced the invasive potential of MDA-MB-231 cells through the Matrigel (Figure 5c). Quantification of results of matrigel invasion revealed a significant reduction of invasive potential of MDA-MB-231 cells when subjected to treatment with pCD (58%) compared to pSV control, which set as100% (Figure 5d).

Knockdown of CD151 expression by RNAi reduces ECM-mediated cell adhesion

Adhesion of cancer cells to ECM components is required to perform key functions such as proliferation, cell cycle, migration, and tumour invasion [34,35]. Treatment with pCD for 48 h reduced adhesion of MDA-MB-231 cells in the order of collagen I, vitronectin, fibronection and laminin (Figure 5e). The percentage of reduction of cell adhesion to collagen I was 60%, fibronectin was 48%, vitronectin was 45% and laminin was 12% (Figure 5f). These results indicate significant reduction of MDA-MB-231 cell adhesion to collagen I followed by fibronectin by down regulation of CD151 with pCD.

Discussion

CD151, a member of tetraspanin superfamily forms tetraspanin-enriched microdomains (TEM) with various signalling molecules [36,37], membrane receptors and other tetraspanins such as CD9, CD81 and CD63. CD151 associate with these proteins and serve as molecular facilitator [38]. Previous studies have shown that many cancer cell lines and cancer tissues had high expression of CD151 and related to poor prognosis [11]. Recent scientific research has verified correlation between CD151 expression and cancer development [39]. Evidence also supported contribution of CD151 in cancer metastasis [40].

Proteomics helps cancer research with aid of high-throughput screening of biomarkers and therapeutic goals. The biological properties of a protein molecule depend on its physical interaction with other molecules. In the present study, interaction partners with CD151 as a central molecule were retrieved from STRING database [41]. The analysis of data showed that CD151 exhibited interactions with high confidence score of 0.900 and functions in different cellular, biological and molecular processes. This study suggests that CD151 cooperates with other proteins as a part of functional module and deliver various biological functions [42]. Previously, Ranjan Prasad Devbhandari et al. used large-scale proteomics screening techniques to develop a map of extensive protein-protein network centered on CD151 [43]. Laetitia Zona et al. identified a subset of TEM protein association network of 169 proteins with HRas and CD81 using STRING analysis [44].

According to annotation from GO database, CD151 function

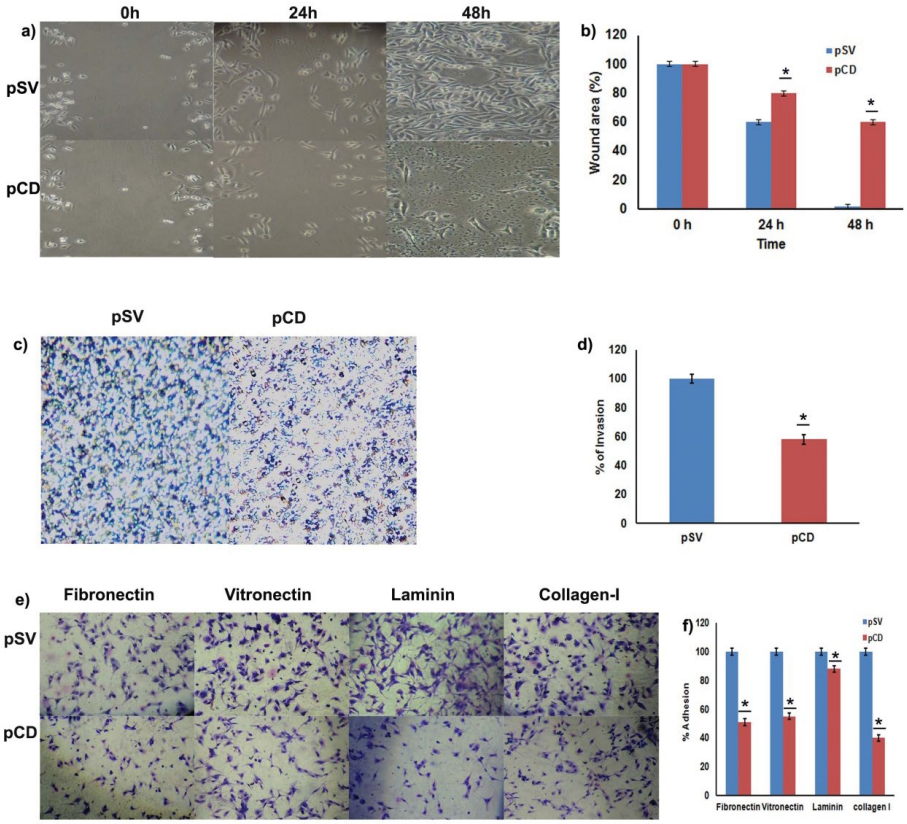

Figure 5: Effect of CD151 gene knockdown on metastasis of MDA-MB-231 cells. a) Effect of pCD on migration potential of MDA-MB-231 cells in Wound scratch assay. Cells were transfected with pSV and pCD for 48h, wound was made and wound healing potential was observed at 0, 24 and 48 h. b) Quantification of wound healing ability of MDA-MB-231 cells. Results were expressed as percent control. c) Effect of pCD on invasion potential of MDA-MB-231 cells through matrigel was evaluated using Boyden chamber assay. d) Quantification of invaded cells was expressed as percent control. e) Effect of pCD on adhesion of different ECM components was evaluated at 48h. Non-adherent cells were washed off and adherent cells were stained with Hema-3 staining. f) Adhesion of MDA-MB-231 cells transfected with CD151 shRNA to collagen-I was reduced compared to other ECM components, Percent of adhesion was calculated by setting adhesion of pSV treated cells as 100%. The results of each experiment was obtained from three independent experiments and values shown as means ±SEM (n=3, *p<0.001).

in receptor binding, adhesion molecular binding and involved in extracellular matrix organization, hemidesmosome assembly and cellular component organization, cell junction assembly related to cancer progress [45]. Further, annotation of CD151 to tissues or to disease entities predicted that CD151 associate with breast glandular cells and breast cancer.

Further, CD151 expression pattern across diverse normal and tumour tissues, tissue sub-types and cancer cell lines was predicted using GENT database. The results demonstrated high expression of CD151 in breast tumour tissues compared to normal tissues. MDA-MB 231 cell lines was selected for CD151 gene silencing study based on information obtained from cell line database. This study predicted that MDA-MB-231 is a good cell line to demonstrate CD151 as a marker. For CD151 functional studies, we used two distinct but prototypical, myoepithelial-basal derived mammary cell lines, such as immortalized normal MCF-12A and malignant MDA-MB-231. The present study demonstrated the over expression of CD151 in triple negative breast cancer cell line, MDA-MB-231 using RT-PCR and Western blot analysis. However, non-cancerous breast epithelial cell line, MCF-12A expressed undetectable levels of this protein. These results are consistent with previous reports [46]. The potential relevance of CD151 with relative selectivity in cancers rather than normal tissues, it may consider as high-priority therapeutic target.

RNA interference is a biological mechanism by which double-stranded RNA (dsRNA) knockdown genes by targeting complementary mRNA. It is breakthrough in understanding regulation of gene expression in cells. RNA interference mediated gene silencing is tested in clinical trials for a number of diseases [47,48]. It also represents as an approach for drug discovery and development. Treatment of cancers using small interfering RNA (siRNA) has been a promising approach and actively explored as an alternative to chemotherapy [49,50]. The shRNA mediated RNAi has become a powerful tool for its specificity and efficiency to knock-down targets that cannot be readily down-regulated by conventional chemotherapy.

pSilencer 2.1-U6 puro vector is most efficient transduction tool for the long-term study of gene specific knockdown (Ambion, Austin, TX, USA). In this study, linearized *pSilencer* 2.1-U6 puro was used for construction of vector encoding a small hairpin RNA (shRNA) directed against human CD151. The advantage of using shRNA over chemically synthesised siRNAs includes stable gene expression and long-term gene silencing effects with minimal cytotoxicity [51,52]. This vector has human U6 RNA pol III promoter, which is more effective for siRNA and provide high levels of constitutive expression across variety of cell types [53].

CD151 shRNA vector was efficiently transfected in to MDA-MB-231 cells using Lipofectamine® 3000 (Life Technologies, USA),

which significantly silenced CD151 expression at mRNA level. MTT assay was originally used to measure the cytotoxic effect of drugs. The amount of formazan produced is proportional to metabolically active cells, which in turn indicates viability of the cells. CD151 gene knockdown via shRNA resulted significant decrease in viability of MDA-MB-231 cells compared to MCF-12A cells. These results were in concordance with the studies investigated the cytotoxic effect of CD151 towards human gastric carcinoma [9]. CD151 silencing using shRNA is neither affected proliferation nor survival of MCF-10A cells [10]. However, CD151 gene silencing via lentivirus-mediated CD151 specific shRNA arrested cell cycle in Go/G1 phase in MCF-7 and MDA-MB-468 cells [54].

Adhesion of MDA-MB-231 cells to various ECM components plays a major role in tumour invasion cascade. Several studies have reported that CD151 form tight complexes with laminin binding integrins that promote tumour cell migration, invasion and proliferation [55]. CD151 in association with MMP-7 initiate cancer invasion and metastasis [56,57]. In the present study, CD151gene silencing caused significant reduction of MDA-MB-231 cell adhesion to collagen-I and moderate reduction to other ECM components. CD151 knockdown using shRNA significantly inhibited invasion of MDA-MB-231 cells through matrigel. Knockdown of CD151using shRNA has caused considerable inhibition of adhesion compared to other cancers [58]. Recent study have shown that knockdown of CD151 has a profound inhibitory effect on metastasis of MDA-MB-231 breast cancer cells [59].

CD151gene silencing significantly inhibited wound healing in wound scratch assay. In contrast, over expression of CD151 significantly increases intercellular adhesiveness and delays wound closure in a scratch assays [60]. Pengcheng et al. demonstrated that CD151-siRNA significantly decreased the cellular proliferation, migration, invasion and colony formation and enhanced apoptosis in lung cancer cell line, A549 [61]. Other studies have revealed that knockdown of CD151 by short-hairpin RNA decreased the cell motility, tumourigenicity, cell migration and decreases experimental metastasis [62] thus emphasizing CD151 as a potential marker [7]. As apoptosis has become a new therapeutic target in cancer research, our results found that knockdown of CD151 expression significantly induced apoptosis in MDA-MB-231 cells as demonstrated by a dramatic increase of TUNEL-positive cells. However, further investigation of its activity is necessary to elaborate and exploit this promise.

The present study conclude that CD151 is positively associate with the metastasis of triple negative breast cancer cell line, MDA-MB-231 and consider as a novel marker for predicting the prognosis of breast cancer patients and may be potential therapeutic targets.

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