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Nectin-4 is a breast cancer stem cell marker that induces WNT/β-Catenin signaling via Pi3k/Akt axis

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Short title: Nectin-4, a breast cancer stem cell marker

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Highlights

- Nectin-4 is a marker for breast cancer stem cells.
- Nectin-4 upregulates, EMT, metastasis and WNT/β-Catenin pathway via Pi3k/Akt axis.
- Nectin-4 depletion inhibited EMT, metastasis, invasion and WNT/β-Catenin pathway.
- Clinicopathological data show induced Nectin-4 in breast tumor metastases to axillary lymph node.

ABSTRACT

Nectin-4 is well known as a junction protein. Recent reports have implicated it in cancer, but there has been little exploration of its functional significance in metastasis and cancer stem cells. Here, using the breast cancer metastasis model system, we report Nectin-4 is a marker for breast cancer stem cells (BCSCs) and provide experimental evidence suggesting that it utilizes WNT/β-Catenin signaling via Pi3k/Akt axis for self renewal of BCSCs. In vitro, in vivo, ex vivo and clinical pathological data showed upregulated Nectin-4 in breast cancer metastasis and WNT/β-Catenin signaling. Nectin-4 depletion inhibited EMT, metastasis, invasion, and the WNT/β-Catenin pathway; conversely, Nectin-4 overexpression in null cells upregulated EMT and metastasis and also induced WNT/β-Catenin signaling via Pi3k/Akt axis, which in turn, controls cancer stem cell proliferation. Induced Nectin-4 was observed in breast tumor patient samples and in breast tumor metastases to axillary lymph nodes, which indicated that Nectin-4 is not only a BCSC marker but also a breast cancer metastasis marker. The current study provides clear evidence that Nectin-4 is a BCSC marker and is responsible for breast cancer metastasis.

Keywords: Nectin-4, cancer stem cells, metastasis, WNT/β-Catenin, Pi3k/Akt
1.0 Introduction

Cancer stem cells (CSC) represent rare, quiescent population of cells present in the bulk of tumor responsible for tumor invasion, migration, cancer relapse, self-renewal and drug resistance (Chiou et al., 2008; Costea et al., 2006; Dalerba et al., 2007; Jordan et al., 2006; Locke et al., 2005). A number of cell surface markers are being used for the isolation of a subset of population enriched in CSCs and for the study of EMT (e.g. CD44, CD24, ALDH1, CD133, E-Cadherin, Vimentin, Oct-4, Nanog, etc) (Steinestel et al., 2014; Wang et al., 2013). Antibody based therapy of above markers with malignant tumor may not be effective due to various mechanisms such as the probability of target receptor saturation kinetics, inappropriate dosage, physical properties, pharmacokinetics and heterogeneous expression of the target antigen (Scott et al., 2012). In view of this, there is a need for the time to search for new CSC markers.

We recently established a breast cancer metastasis model using different breast cancer cells as well as tumor derived primary cells and reported the existence of a novel, intermediate, adherent pre-metastatic phase characterized by Survivin (Siddharth et al., 2016a). Athanassiadou et al. showed a strong correlation between the expression levels of Survivin and Nectin-4 in breast carcinoma (Athanassiadou et al., 2011).

Nectins represent a family of immunoglobulin like cell adhesion molecule crucial for the formation and maintenance of Cadherin based adherens and Claudin (recently reported as a CSCs marker (Blanchard et al., 2013)) based tight junctions. Nectin-4 (NCBI Gene ID: 81607) has been reported as a cancer biomarker as it is expressed in cancer tissues (eg. ovary, breast,
lung, bladder and cervical) and cancer cell lines but not in normal cells (Derycke et al., 2010; Fabre-Lafay et al., 2005; Takano et al., 2009). Nectin-4 promotes anchorage independent cell growth and plays a role in cell-cell adhesion; activates Pi3k-Akt signaling by interacting with Afadin (Pavlova et al., 2013; Takai et al., 2008). Reports also revealed that Nectin-4 promotes gallbladder metastasis in vitro and in vivo (Zhang et al., 2016).

We have already reported that Nectin-4 induces Pi3k/Akt cell proliferation pathway and imparts chemoresistance to colon cancer cells against 5-FU (Das et al., 2015). We have also reported WNT activation as the functional self-renewal pathway for CSCs in an in vitro and ex vivo breast cancer metastasis model (Siddharth et al., 2016a). The present work reports Nectin-4 to be a probable marker for breast CSCs and it upregulates WNT signaling (a self-renewal pathway of CSCs) via Pi3k/Akt axis.

2.0 Material and methods

2.1. Cell culture, development of metastasis model and reagents

Breast cancer (MDA-MB-231, MCF-7, MDA-MB-468 and T47D) and cigarette smoke transformed (MCF-10A-Tr) or simply (Tr) cells were grown and cultured in DMEM supplemented with 10% FBS, 1.5 mM L-glutamine and 1% antibiotic (100 U/ml of penicillin, 10 mg/ml of streptomycin) at 37°C in a humidified atmosphere of 5% CO₂. Metastasis model was developed according to the protocol mentioned earlier (Siddharth et al., 2016a). In brief, Tr cells were trypsinized and resuspended in serum free hypoxic media supplemented with 10 ng/ml basic fibroblast growth factor (bFGF), 20 ng/ml epidermal growth factor (EGF), 5 µg/ml insulin, 0.4% bovine serum albumin (BSA) and 200 µM CoCl₂ for mammosphere formation. Instead of mammosphere formation, initially the cells formed a monolayer, stable till 7 passages; we
termed it as Quiescent Tr (Q-Tr). Prolong culture of Q-Tr cells under similar conditions formed mammospheres (MAMMO) which then formed a stable monolayer of adherent immortal cell line (P-EMT) when introduced in serum endowed conditions. Cell culture reagents and other growth supplements were procured from Himedia, India. Nectin-4 siRNA (#sc-62669) and scrambled siRNA (#sc-37007) was purchased from Santa Cruz Biotechnology Inc., CA, USA. Anti- Nectin-4 (ab57873) and anti-CD44 (ab23557) were purchased from Abcam, MA, USA. Anti-Akt (#9272), anti-Pi3k (#4292), anti-β-Catenin (#9587), anti-E-Cadherin (#3195) and anti-Vimentin (#5741) were purchased from Cell Signaling Technology, MA, USA. Anti-GAPDH (sc-25778) was purchased from Santa Cruz Biotechnology Inc., CA, USA. Anti-CD-133 (AC133) was purchased from MACS, Miltenyi Biotec Asia Pacific Pte Ltd.

2.2. Development of an ex vivo metastatic model system from breast tumor sample

Breast tumor sample was collected from Acharya Harihar Regional Cancer Centre, Cuttack, Odisha, as per the Hospital Review Board, under ethical guidelines of the hospital. Tumor tissue was processed for the formation of the metastasis model following the protocol mentioned earlier (Siddharth et al., 2016a). In brief, tumor tissue in DMEM without phenol red supplemented with 10% FBS, 2x MEM non-essential amino acid solution, a mixture of 0.14 mM ampicillin, 0.26 µM Amphotericin B, 7.54 µM ciprofloxacin was chopped to pieces and then dissociated using an enzyme cocktail of collagenase and neutral protease at 37°C for 2h with rotation. The cells were sieved sequentially through 100 and 40 µm cell strainer and 1 x 10^6 cells were directly sorted for CD44+/CD24-, CD44+/CD24+, CD44-/CD24+ and CD44-/CD24- in 1 ml DMEM containing 10% FBS, 1.5 mM L-glutamine and 1% antibiotic (100 U/ml penicillin and 10 mg/ml streptomycin) using FACS Aria II and then seeded onto 60 mm dishes. Cells were allowed to
grow for 7 days after which the cells were trypsinized and processed for metastasis model development following the protocol mentioned earlier (Siddharth et al., 2016a).

2.3. **Surface staining by FACS**

Trypsinized cells were washed twice with 1X PBS, followed by blocking with 2% BSA containing 0.2% Triton X-100. Then, the cells were incubated with anti-Nectin-4 antibody in dark at 4°C for 45 min. After washing twice with 1X PBS, secondary anti-mouse FITC conjugated antibody (1:750) was added and incubated at 4°C for 30 min. Then, the cells were washed and 10,000 gated cells were analyzed by FACS Calibur (Becton Dickinson Biosciences, San Jose, USA) using Cell Quest Pro software.

2.4. **Immunostaining of Nectin-4**

Fixation of cells grown on cover slips in 24 well tissue culture plates and mammospheres grown in 6 well ultra low attachment plates was done with pre-chilled acetone : methanol (1:1) for 20 min at -20°C (mammospheres were fixed onto polystyrene coated slide). Then, cells were washed twice with 1X PBS and incubated overnight with anti-Nectin-4 antibody at 4°C. Unbound antibodies were removed by washing twice with 1X PBS. Secondary anti-mouse FITC conjugated antibody was added and incubated for 2 h at room temperature (RT). Nuclei were counter stained with Propidium Iodide (PI) (0.5µg/mL). Immunofluorescence was detected using fluorescence microscope (Evosfl Fluorescence Microscope, Thermo Fisher Scientific, MA, USA) at 40X magnification.

2.5. **ELISA**
Detection of soluble and serum markers (Nectin-4, E-Cadherin, Vimentin and CD44) was done by Indirect ELISA according to the protocol mentioned earlier (Siddharth et al., 2016a). In brief, the protein antigen mixed in coupling buffer was coated onto 96 well microplate (3679, Corning, NY, USA) and incubated overnight at 4°C followed by washing with 1X PBST and then blocking with super cocktail buffer. Then, primary antibodies were added and incubated for 2 h at RT. After washing thrice with 1X PBST, the wells were incubated with secondary HRP linked antibody for 45 min at RT followed by washing thrice with 1X PBST. Then, 2, 2′-azinobis (3-ethylbenzthiazoline-6-sulfonic acid) substrate solution was added, incubated for 10 min in dark and absorbance was read at 405 nm using microplate reader (Berthold, Germany).

2.6. mRNA expression of nectin-4

Cells of respective stages were harvested and the total RNA was isolated using GeneJET RNA Purification Kit (Cat No. K0732, Fermentas, USA). The isolated RNA was used for cDNA synthesis using RevertAid First Strand cDNA Synthesis Kit (Cat No. K1622, Fermentas, USA). Gene specific amplification of nectin-4 and GAPDH was done by PCR (Eppendorf, Germany). Sequences for the primer were as follows: nectin-4, 5′- CAAAATCTGTGGCACATTGG -3’ (forward) and 5′- GCTGACATGGGCAGACGTAGA -3’ (reverse); GAPDH, 5′- AGAAGGCTGGGGCTCATTTG- 3’ (forward) and 5′- AGGGGCCATCCACAGTCTTC- 3’ (reverse). All the primers were purchased from Integrated DNA Technologies, USA. The samples were separated on 0.9% agarose gel after amplification. The band intensity of each lane was measured using a UVP Imaging system (UVP, Cambridge, UK).

2.7. Western blotting
Western blot analysis was performed according to the protocol mentioned earlier (Siddharth et al., 2016b). Whole cell lysates were prepared and processed for western blot analysis according to the antibody specific manufacturer’s protocol. To detect the expression level of different proteins in the tumor lysate, western blotting was performed after lysis of the breast fat pad following the protocol mentioned earlier (Mohapatra et al., 2014). Numerical values above every panel of protein represents the band intensity of each lane measured using a UVP GelDoc-It ® 310 Imaging system (UVP, Cambridge, UK).

2.8. **Co-localization of Vimentin and E-Cadherin in the tumor xenograft**

Frozen sections of tumor xenograft were fixed in chilled 70% ethanol for 20 minutes at -20 °C followed by blocking with 10% FCS (Foetal calf serum). Then, the slides were incubated with anti-Vimentin and anti-E-Cadherin at 1:750 dilutions overnight. Then, slides were washed with 1X PBS and incubated with secondary anti-Rabbit-TRITC (for Vimentin) and anti-Mouse-FITC (for E-Cadherin), respectively, at 1:1000 dilutions followed by PBS wash. Nuclei were counterstained with DAPI and immunofluorescence was detected on mounted slides using fluorescence microscope (Evosfl Fluorescence Microscope, Thermo Fisher Scientific, MA, USA) at 40X magnification.

2.9. **Knockdown of Nectin-4 in Tr cells**

Wild type gene expression of Nectin-4 was knocked down using Nectin-4 siRNA following the protocol mentioned earlier (Das, et al., 2015). Cells were grown on 60 mm tissue culture discs to 60% confluency followed by transfection with 0.25 µg of Nectin-4 siRNA or scrambled siRNA (control) with Lipofectamine 2000® (Cat. No. 11668-027) following manufacturer’s (Invitrogen, Thermo Fisher Scientific, Waltham, Massachusetts, USA) protocol.
2.10. **Over-expression of p3XFLR4.C1 nectin-4 plasmid in MDA-MB-231 cells**

Nectin-4 was overexpressed in MDA-MB-231 cells with 4µg3XFLR4.C1nectin-4 plasmid using Lipofectamine 2000® transfection reagent following the protocol mentioned earlier (Das et al., 2015). Overexpression of Nectin-4 was detected by western blot analysis.

2.11. **Development of breast tumor xenograft in female Balb/c mice**

Female *Balb/C* mice were housed in 12h/12h proper light/dark cycle at KIIT School of Biotechnology, KIIT University, Bhubaneswar, Odisha, India. The animal work and experimentation protocols were duly approved by the Institutional Animal Ethical Committee (IAEC, KIIT School of Biotechnology, KIIT University, Bhubaneswar, Odisha, India). Tr,Q-Tr,MAMMO,Nectin-4 silenced Tr,MDA-MB-231,Nectin-4 overexpresssed MDA-MB-231 and the metastasis model developed from the overexpressed cells, respectively, (1x10⁷cells in 200 µl of freshly prepared PBS) and an equal volume of PBS were implanted in the left mammary fat pad of different groups of mice (6 mice each group). The mice were monitored every other day and after 25 days, the mice were euthanized and the tumor tissues were collected for H&E staining, western blotting, ELISA and IHC.

2.12. **H&E staining**

H&E staining was performed following the protocol described previously (Nayak et al., 2016). Using Shandon cryotome FSE (Thermo scientific), the frozen tissues (both human and mice breast fat pad samples) were cut into 4 µm sections and mounted on SuperFrost® plus slides (Thermo Scientific) at -30°C. The slides were then immersed in different percentage (100% to 50%) of alcohol for rehydration. Dried slides were dipped into hematoxylin followed by eosin (H&E) stain. Then, the slides were dehydrated by immersing in increasing percentages of
alcohol (50% to 100%). After 2 minutes of incubation in xylene, images were captured in bright field microscope using 20X magnification (Leica DM200, USA).

2.13. **Immunohistochemistry of Nectin-4**

Fixed slides (both human and mouse tissue) were blocked in 100µl blocking solution (5% BSA) followed by peroxide blocking. Then, anti-Nectin-4 antibody (1:750) was added and incubated overnight at 4°C. Slides were then washed with 1X PBST and incubated with HRP conjugated secondary antibody (1:750) for 60 min at RT. The slides were developed using DAB peroxidase substrate kit (SK-4100, Vector Laboratories, CA, USA). Images were captured in 20X magnification using brightfield microscope (Leica DM2000, USA).

2.14. **Sphere formation assay**

The sphere formation assay was performed according to the protocol described previously (Siddharth et al., 2016a). Briefly, 1x10³ MDA-MB-231 and MDA-MB-231 Nectin-4 overexpressed cells were plated on a 12 well plate in 500 µl of the serum free DMEM-F12 media containing 10ng/ml bFGF, 20ng/ml EGF, 5µg/ml insulin and 0.4% BSA. 50 µl of fresh medium was added every 5 days and after 20 days, the number of spheres per well was microscopically evaluated at 5 different fields. Data were represented graphically as average number of mammospheres versus number of days.

2.15. **Matrigel invasion assay**

The matrigel invasion assay was performed following the protocol mentioned earlier (Siddharth et al., 2016a) using a 24 well transwell plate (#3422, Corning, NY, USA) with a pore size of
8µm. 20 µl of matrigel (356234, BD Biosciences, CA, USA) was coated onto the inserts and 3x10^5 cells (Tr, Nectin-4 KD, MDA-MB-231, Nectin-4 overexpressed MDA-MB-231 and different stages of metastasis developed from overexpressed cells) were seeded either on uncoated inserts (migration) or on the matrigel coated inserts (invasion) in serum free media. Complete serum containing media was added to the lower chamber and the plate was incubated for 24 h. A cotton swab was used to remove the non-invaded cells followed by fixation of invaded cells with 4% paraformaldehyde. The invaded cells were stained with DAPI and microscopically counted at 5 different fields at 40X under the fluorescence microscope (Nikon, Japan). Data were represented as % invasion against cell types which was calculated using the following formula

\[
\% \text{ Invasion} = \frac{\text{Number of cells invaded the matrigel coated inserts towards the complete media}}{\text{Number of cells migrated the uncoated inserts towards the complete media}} \times 100
\]

2.16. TCF/LEF promoter activity assay

A luciferase-based reporter assay was carried out to measure the promoter activity of the TCF/LEF (WNT transcription factor) as mentioned earlier (Preet et al., 2013). Cells were grown to 70% confluency prior to transfection. Cells were cotransfected with 2.5 µg TOP Flash, a synthetic luciferase-based promoter plasmid (sensitive to the activity of the β-catenin/TCF-4 complex, containing three copies of the TCF-4 binding site upstream of a firefly luciferase reporter gene) and β-galactosidase (β-gal) (0.5 µg) using the Lipofectamine 2000® transfection reagent. Next, an equal amount of the mutant form of the above promoter (FOP Flash) and β-gal were cotransfected using the Lipofectamine 2000® transfection reagent. β-gal was used to monitor the efficiency of transfection in the cells. Similarly in another set of cells nectin-4 was
silenced by si-RNA treatment followed by transfection with TOP Flash/FOP Flash plasmid and β-gal and incubated for 24h post transfection. Cells were then lysed, and efficiencies of transfection were normalized to β-gal activity. Luciferase activity was measured by microplate reader (multimode ELISA reader). The relative luciferase activity (TOP Flash/FOP Flash) was calculated from triplicate experiments.

2.17. Statistical significance

The statistical significance was performed using Graph Pad Prism version 5 software, USA. The results were expressed as mean ± S.E.M. of 5 different experiments. The data were analysed by repeated measure one-way analysis of variance (ANOVA) followed by Bonferroni’s multiple comparison test. The statistical significance of difference in the central tendencies was designated as *p < 0.05, ** p < 0.005 and *** p < 0.001.

3.0. RESULTS

3.1. Nectin-4 is overexpressed in an in vitro breast cancer metastasis model

We already established a metastatic model of breast CSCs and found upregulated Nectin-4 expression by western blot analysis (Siddharth et al., 2016a). To further validate our western result, we examined Nectin-4 expression by FACS, ELISA, semi-quantitative PCR and fluorescence microscopy. An increasing pattern of Nectin-4 expression in Q-Tr (10.30%), MAMMO (20.19%) with a maximum of 36.18% in P-EMT was noted in Nectin-4-FITC stained cells compared to Tr (2.96%) by FACS (Fig 1A). Expression of soluble Nectin-4 increased to 2 and 2.5 fold in MAMMO and P-EMT cells, respectively, with a minor increase in Q-Tr compared to Tr in culture supernatant (Fig 1B). Interestingly, maximum increased mRNA expression of Nectin-4 was noted in P-EMT (> 9 fold compared to Tr), followed by MAMMO (>
6 fold compared to Tr) and Q-Tr (> 4 fold compared to Tr) (Fig 1C). Fig 1D demonstrated the increased level of Nectin-4 inside the cells in different stages of metastasis with maximum expression in P-EMT, followed by MAMMO and Q-Tr compared to Tr.

3.2. Administration of metastatic cells into Balb/C mice caused elevation of Nectin-4 expression, induction of EMT and WNT activation via Pi3k/Akt axis

We have already reported the tumor forming ability of Tr cells in Balb/C mice (Mohapatra et al., 2014). Here, we wanted to study whether the cells of different phases of metastasis possess tumor forming ability in Balb/C mice and the expression of different proteins related to BCSCs, EMT and WNT signaling. H&E staining revealed large, deformed, irregularly distributed and accumulated nuclei compared to PBS control group (Fig 2A). IHC data showed increased Nectin-4 expression (indicated by arrow) in metastatic cells implanted mice compared to PBS control (Fig 2B). Serum CD44 and Nectin-4 increased to a maximum of 9 fold in P-EMT, followed by 7 fold in MAMMO, more than 5 fold in Q-Tr and 2 fold in Tr implanted mice compared to PBS control. CD133 also followed a similar pattern of increase with a maximum of 9 fold in P-EMT, followed by 8 fold in MAMMO and more than 5 fold in Q-Tr compared to PBS control (Fig 2C). We also found activated Pi3k, Akt, Nectin-4 and β-Catenin in the metastatic model of Tr (Siddharth et al., 2016a). We showed the complete abolishment of E-Cadherin with the simultaneous upregulation of Vimentin and N-Cadherin in MAMMO compared to other stages of metastatic model of Tr (Siddharth et al., 2016a). Here, we wanted to validate our in vitro data with our in vivo observation. So, we performed a western blot analysis of the whole cell lysate of tissue sample collected from metastatic cells implanted breast fat pad of female Balb/c mice. Fig 2D demonstrated more than 3 fold activated Pi3k in P-EMT followed by 2 fold activation in MAMMO and 1.5 fold in Q compared to Tr implanted cells. 5 fold
activated Akt in P-EMT followed by 2 fold activation in Q and MAMMO was noted compared to Tr implanted cells. Nectin-4 upregulated to 8 fold in P-EMT and 7.9 fold in MAMMO with a minor increase in Q compared to Tr implanted cells. 5 fold and 2.5 fold increased β-Catenin in P-EMT and MAMMO was also noted (Fig 2D). 10 fold downregulation of E-Cadherin with a simultaneous 3 fold activation of Vimentin was noted in MAMMO with an unaltered expression of both in other stages and Tr implanted cells (Fig 2D). To confirm that the decrease of epithelial marker, E-Cadherin coincident with increase in mesenchymal marker, Vimentin signals EMT, we performed a co-localization study of E-Cadherin and Vimentin in the tissue xenograft implanted with metastatic cells. Fig 2E demonstrated that Vimentin is expressed only in the MAMMO implanted xenograft tissue with no expression of E-Cadherin in the same.

3.3. Nectin-4 deletion inhibited invasion, formation of EMT, WNT signaling cascade

The above experiments suggest Nectin-4 to be a marker of breast CSCs and play a significant role in the formation of mammospheres. To understand the precise role of Nectin-4 as a breast cancer stem cell marker more convincingly, Nectin-4 was knocked down in Tr cells using Nectin-4 siRNA and a series of experiments were carried out (Fig 3). We cultured Tr Nectin-4 knockdown (Nectin-4 KD) cells in serum free media for the formation of metastasis model. These Nectin-4 KD cells did not form the intermediate Q-Tr and eliminated the formation of mammospheres (EMT) or lead to formation of non functional spheroids. The deformed spheroids were unable to produce P-EMT stage (Fig 3A). No invasion was observed by matrigel assay (Fig 3B). Unaltered E-Cadherin and 2 fold decrease of Vimentin suggest the potential involvement of Nectin-4 during EMT (Fig 3C). The complete absence of β-Catenin, Pi3k and Akt with a decrease in the reporter activity of WNT-TCF transcription factor in Nectin-4-KD cells provide experimental evidence for the inhibition of WNT signaling cascade via Pi3k/Akt axis in Nectin-4.
absence (Fig 3C and D). Next MCF-10A-Tr and MCF-10A-Tr-Nectin-4 KD cells were implanted into the breast fat pad of female Balb/C mice. Normal, condensed and uniform nuclei were observed similar to the PBS control with no abnormalities (Fig 3E). IHC data showed no Nectin-4 expression (indicated by arrow) in Nectin-4 KD cells implanted Balb/C mice (Fig 3F).

3.4. Nectin-4 overexpression in Nectin-4 null cells formed metastasis model, invaded matrigel, upregulated WNT signaling and elevated Nectin-4 in Balb/C mice

To further confirm the role of Nectin-4 in metastasis, we have overexpressed Nectin-4 in Nectin-4 null cells (MDA-MB-231) (Noyce et al., 2011) by transiently transfecting p3XFLR4.C1nectin-4 plasmid. When vehicle transfected MDA-MB-231 was cultured for the enrichment of CSCs population in serum deprived conditions, the non formation of functional spheroids was noticed which resulted in the non occurrence of Q and P-EMT stage (Fig 4A). But, Nectin-4 transfected cells lead to the formation of Q phase (Q-Nectin-4) as an adherent phase followed by spheroid formation (MAMMO Nectin-4) which resulted in the formation of P-EMT stage (P-EMT Nectin-4) (Fig 4B). Fig 4C represents sphere formation assay with a maximum number of viable mammospheres on 10th day in overexpressed cells with no/least mammospheres detected in normal MDA-MB-231 cells. Increased invasiveness was noted by different metastatic cells formed from Nectin-4 overexpressed MDA-MB-231 (Fig4D). Overexpression of Nectin-4 was detected by western blotting (Fig 4E). Fig 4F represents upregulated WNT-β-Catenin transcriptional activity with highest activity expressed by P-EMT cells, followed by MAMMO and Q cells compared to control. All these results indicated the crucial role of Nectin-4 in breast cancer metastasis. Then, we implanted Nectin-4 overexpressed MDA-MB-231 and the stages developed from it into the breast fat pad of female Balb/C mice. H&E staining revealed cancerous morphology with aggregated, elongated, irregularly distributed nuclei with higher
nuclei to cytoplasmic ratio when overexpressed cells and stages developed from it were implanted into the Balb/C mice compared to MDA-MB-231 implanted mice (Fig. 4G). Higher expression of Nectin-4 was detected by IHC (indicated by arrow) in overexpressed cells and stages developed from it implanted mice while no Nectin-4 was detected in the MDA-MB-231 implanted mice (Fig. 4H).

3.5. Nectin-4 detection in breast tumor samples and WNT activation via Pi3k/Akt in breast tumor derived primary cells

To assess the clinical implication of Nectin-4 in breast cancer cases, we performed H&E staining and IHC in breast tumor samples. Data from H&E staining showed normal adipose tissue in the cut margin while aggregated nuclei with higher nuclei to cytoplasmic ratio in the tumor sample (Fig. 5A). H&E data of breast tumor metastasis to axillary lymph node indicated a cancerous phenotype (Fig. 5A). Immunohistochemistry data revealed highest Nectin-4 expression (indicated by arrow) in breast tumor metastasis to axillary lymph nodes followed by breast tumor samples with least/no Nectin-4 in the cut margin (Fig. 5B). We established a metastasis model from CD44+/CD24- population of breast tumor derived primary cells as described in materials and methods following the protocol mentioned earlier (Siddharth et al., 2016a) and studied the expression profile of different proteins involved in WNT signaling along with Nectin-4 in the metastasis model of tumor derived primary cells. CD44+/CD24+, CD44-/CD24+ and CD44-/CD24- sorted cells failed to form the metastasis model. Fig. 5C demonstrate an 8 fold upregulated β-Catenin in tumor derived PEMT cells followed by 5 fold and 4 fold in MAMMO and Q cells compared to control. More than 4 fold activated Pi3k was noted in tumor derived P-EMT and MAMMO cells followed by 2 fold activation in Q cells compared to control. Akt activated to more than 5 fold in P-EMT and MAMMO cells followed by approximately 4 fold
activation in Q cells in compared to control. It was observed that Nectin-4 upregulated to 8.5 fold, 6.9 fold and 5.3 fold in P-EMT, MAMMO and Q cells compared to control (Fig 5C).

3.6. Activated Nectin-4 induced WNT signaling is the functional self renewal pathway in metastatic model of different breast cancer cells

We have already established the metastasis model of different breast cancer cells (MCF-7, MDA-MB-468, T47D and ZR75-1) (Siddharth et al., 2016a). Here, we studied the role of Nectin-4 induced WNT cascade in different breast cancer metastasis model (Fig 6). Results reveal more than 3 fold activated Nectin-4 with simultaneous 5 fold activated β-Catenin in 7-P-EMT and 7-MAMMO compared to parental MCF-7 cells (Fig 6A). Fig 6B measures the soluble Nectin-4 expression in culture supernatant by ELISA and demonstrate 4 fold higher soluble Nectin-4 in 7-P-EMT, followed by 3 fold higher in 7-MAMMO compared to MCF-7 cells. Relative luciferase assay of WNT-β-Catenin transcriptional activity revealed highest transcriptional activity by 7-P-EMT, followed by 7-MAMMO and Q-7 cells compared to parental MCF-7 cells (Fig 6C). 4 fold activated Nectin-4 (468-P-EMT and 468-MAMMO) with 2 fold activated β-Catenin (468-P-EMT and 468-MAMMO) compared to parental MDA-MB-468 was observed in the metastatic model of MDA-MB-468 (Fig 6D). Approximately, 4 fold activated soluble Nectin-4 was detected in 468-P-EMT cells, followed by almost 3 fold activation in 468-MAMMO compared to parental MDA-MB-468 (Fig 6E). The highest relative luciferase activity for WNT-β-Catenin transcription factor (TOP FLASH and FOP FLASH) was detected by the 468-P-EMT cells, followed by 468-MAMMO and Q-468 compared to control (Fig 6F). The metastatic model of T47D demonstrated more than 2.5 fold and 3 fold upregulated Nectin-4 in whole cell lysate and culture supernatant of T47D-P-EMT compared to control (Fig 6G and H). T47D-MAMMO revealed similar of 2.5 fold and 2 fold activated Nectin-4 in whole
cell lysate and culture supernatant compared to control (Fig 6G and H). Fig 6I demonstrated maximum WNT-β-Catenin transcription factor activity by T47D-P-EMT cells, followed by T47D-MAMMO and Q-T47D cells compared to parental T47D cells.

To confirm the role of Nectin-4 in breast cancer metastasis, we silenced Nectin-4 in multiple breast cancer cells and performed western blot and ELISA for total and soluble Nectin-4 detection as well as relative luciferase assay for WNT-β-Catenin activity (Fig 7). Nectin-4 depletion in multiple breast cancer cell lines resulted in downregulation of WNT-β-Catenin pathway and non formation of different metastatic phases (Fig 7A-I).

4.0. DISCUSSION

Despite the involvement of Nectin-4 in cancer biology, there is little information regarding its role in metastasis and cancer stem cells. Recently, we have established a metastasis model of breast cancer stem cells and found an upregulation of Nectin-4 in the metastasis model (Siddharth et al., 2016a). Here, we explored Nectin-4 as a breast cancer stem cell marker and its influence on WNT/β-Catenin signaling. Using multiple means, we found that Nectin-4 was highly expressed in metastasis models (in vitro), in metastatic cells implanted into mice (in vivo), in a metastatic model of breast tumor derived primary cells (ex vivo), and in breast tumor metastasis in axillary lymph node samples compared to non-tumor cut margins (clinical pathology).

Earlier, we reported the activation of the WNT signaling pathway via the Pi3k/Akt axis (Siddharth et al., 2016a) in MCF-10A-Tr breast cancer metastasis model. We and others also reported that Nectin-4 activates Pi3k/Akt cascade and promotes cell survival, proliferation, and growth in colon cancer and gall bladder cancer (Das et al., 2015; Zhang et al., 2016). Here, we
report that Nectin-4 activates the WNT signaling pathway (a self renewal pathway for cancer stem cells) via the Pi3k/Akt axis. Nectin-4 silencing downregulated WNT signaling while overexpression in null cells activated WNT signaling. Clinical pathological data also showed increased Nectin-4 in breast tumor metastasis in axillary lymph nodes. Data from the *ex vivo* metastasis model of tumor derived primary cells also indicate upregulated Nectin-4 and WNT signaling activation via the Pi3k/Akt axis.

Immunotherapy involving antibodies targeting the breast cancer stem cell specific markers is often given in addition to radiation and chemotherapy but is associated with many drawbacks. To overcome these shortcomings, we add Nectin-4 to the list of available breast cancer stem cell and breast cancer metastasis markers. We previously reported that the combination of BCNU and resveratrol induced apoptosis in 5-FU resistant colon cancer cells by decreasing Nectin-4 (Das et al., 2015). So, we propose that both immunotherapy and small molecule inhibitors might be used to target Nectin-4 over expressed cancer stem cells which overcome the limitation of available breast cancer stem cell surface markers.

Endogenous Nectin-4 promotes EMT, metastasis, proliferation and activates WNT signaling pathway and Nectin-4 depletion inhibited EMT, metastasis, proliferation, invasion and WNT signaling. Furthermore the add back of Nectin-4 in null cells not only upregulated EMT and metastasis but also induced WNT signaling via Pi3k/Akt axis which in turn controls cancer stem cell proliferation. Collectively, the observations suggest that Nectin-4 drives breast cancer metastasis. It is also tempting to speculate that the pursuit of anti-Nectin-4 therapy is worthy of further exploration as a molecular-targeted cancer treatment.

5.0. Conclusion
In conclusion, the current study provides a clear insight idea about the potential role of Nectin-4 as breast cancer stem cell marker and suggests that it induces WNT/β-Catenin cascade as the functional self-renewal pathway for breast cancer stem cells.

Acknowledgement

S.S. and C.N.K. conceived the idea, designed the experiments and analyzed the data. S.S. performed the experiments. S.D., A.N., D.N., and C.S. contributed to some experiments. M.D.W. and C.N.K. analyzed data and wrote the manuscript. K.G. provided clinical samples.

Conflict of Interest

None to declare.

References


Figure Legends

**Fig 1: Nectin-4 is over-expressed in breast cancer metastasis.** (A) Expression of Nectin-4 measured by FACS. IgG anti- mouse served as the negative control. (B) ELISA of Nectin-4 in cell culture supernatant. Data shown here is the mean ± SD of three independent experiments. Statistical significance was determined by paired t test. *p < 0.05, ** p < 0.005 and ***p < 0.001. (C) Semi quantitative analysis of Nectin-4 and GAPDH mRNA measured in different stages of metastasis. (D) Cellular expression of Nectin-4 was determined by immunostaining. The images shown here are representative of five independent experiments. Scale bar = 10 µm. Tr: cigarette smoke transformed MCF-10A cells; Quiescent Tr; MAMMO: mammospheres; P-EMT: adherent immortal cell line.

**Fig 2: Metastatic cells implanted xenograft mice revealed Nectin-4 upregulation and WNT signaling activation.** (A) H&E staining of xenograft tissue sections. Scale bar = 20 µm. (B) Immunohistochemical detection of Nectin-4 expression (indicated by arrow). Scale bar = 20 µm. Images are representative of five independent experiments. (C) Indirect ELISA of CD44, Nectin-4 and CD133 in the serum samples of xenograft mice. (D) Expression of proteins in Tr, Q, MAMMO and P-EMT implanted mice tumor lysate by Western blotting. GAPDH served as the loading control. (E) Co-localization of Vimentin-TRITC (2nd panel from left hand side) and E-Cadherin-FITC (3rd panel from left hand side) was determined by immunostaining of the xenograft tissue section. The images shown here are representative of three independent experiments. Scale bar = 10 µm.

**Fig 3: Nectin-4 deletion inhibited invasion, formation of EMT and WNT signaling cascade.** (A) Morphology of Tr in the presence and absence of Nectin-4. Scale bar = 10 µm. (B) Matrigel
invasion assay. (C) Western blot analysis. GAPDH served as the loading control. (D) Relative luciferase activity of WNT transcription factor TCF/LEF. (E) H&E staining of xenograft tissue sections. Scale bar = 20 µm. (F) Immunohistochemical detection of Nectin-4 expression (indicated by arrow). Scale bar = 20 µm. Images are representative of five independent experiments.

**Fig 4:** Nectin-4 overexpression in MDA-MB-231 cells formed the metastasis model, upregulated WNT signaling and elevated Nectin-4 in Balb/C mice. (A) Non formation of metastasis stages in vehicle transfected MDA-MB-231 cells. Scale bar = 10 µm. (B) Different stages of metastasis in MDA-MB-231 cells overexpressed with p3XFLR4.C1nectin-4. Scale bar = 10 µm. (C) Mammosphere formation assay. (D) Matrigel invasion assay. (E) Western blot analysis of Nectin-4 in MDA-MB-231 overexpressed cells. GAPDH served as the loading control. (F) Relative luciferase activity of WNT transcription factor TCF/LEF. (G) H&E staining of xenograft tissue sections. Scale bar = 20 µm. (H) Immunohistochemical detection of Nectin-4(indicated by arrow). Scale bar = 20 µm. Images are representative of five independent experiments.

**Fig 5:** Nectin-4 detection in breast tumor samples and WNT activation via Pi3k/Akt in breast tumor derived primary cells. (A) H&E staining of a breast cancer patient sample. Scale bar = 20 µm. (B) Immunohistchemical detection of Nectin-4 (indicated by arrow) expressed in breast tumor and in breast tumor metastasis to axillary lymph nodes. Scale bar = 20 µm. Images are representative of five independent experiments. (C) Western blot analysis of β-Catenin, Pi3k, Akt and Nectin-4 in the breast tumor derived primary cells. GAPDH served as the loading control.
Fig 6: Nectin-4 induced WNT/β-Catenin pathway in the metastatic model system of different breast cancer cells. Data in Figures 6A-C are from the MCF-7 metastatic model. Data in Figures 6D-F are from the MDA-MB-468 metastatic model. Data in Figures 6G-I are from the T47D metastatic model. (A) Expression of Nectin-4 and β-Catenin in the MCF-7 metastatic model. GAPDH served as the loading control. (B) Detection of soluble Nectin-4 by ELISA. (C) Relative luciferase activity of WNT transcription factor TCF/LEF. (D) Expression of Nectin-4 and β-Catenin in the MDA-MB-468 metastatic model. GAPDH served as the loading control. (E) Detection of soluble Nectin-4 by ELISA. (F) Relative luciferase activity of WNT transcription factor TCF/LEF. (G) Expression level of Nectin-4 and β-Catenin in the T47D metastatic model. GAPDH served as the loading control. (H) Detection of soluble Nectin-4 by ELISA. (I) Relative luciferase activity of WNT transcription factor TCF/LEF.

Fig 7: Nectin-4 deletion in multiple breast cancer cells prohibited the metastasis model formation by downregulating the WNT cascade. Data in Figures 7A-C are from the MCF-7 metastatic model. Data in Figures 7D-F are from the MDA-MB-468 metastatic model. Data in Figures 7G-I are from the T47D metastatic model. (A) Expression of Nectin-4 and β-Catenin in MCF-7 and MCF-7 Nectin-4 silenced cells. GAPDH served as the loading control. (B) Detection of soluble Nectin-4 by ELISA. (C) Relative luciferase activity of WNT transcription factor TCF/LEF. (D) Expression of Nectin-4 and β-Catenin in MDA-MB-468 and MDA-MB-468 Nectin-4 KD cells. GAPDH served as the loading control. (E) Detection of soluble Nectin-4 by ELISA. (F) Relative luciferase activity of WNT transcription factor TCF/LEF. (G) Expression level of Nectin-4 and β-Catenin in T47D and T47D Nectin-4 KD cells. GAPDH served as the loading control. (H) Detection of soluble Nectin-4 by ELISA. (I) Relative luciferase activity of WNT transcription factor TCF/LEF.
Fig 3

A) Tr and Nectin-4 KD

B) No. of cells invaded the matrigel

C) Control vs Nectin-4 KD

D) Relative Luciferase Activity (TOP FLASH/FOP FLASH)

E) Histological images

F) 1X PBS, MCF-10A-Tr, Nectin-4 KD