**Clinical significance of Nectin-4 expression in metastasis and angiogenesis for tumor relapse**

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**Running tile –** Nectin-4 expression in metastasis and angiogenesis

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**Materials and Methods:**

**Histopathological study (H&E staining) and Immunohistochemical (IHC) analysis**

Paraffin-embedded specimens were sectioned at 5 µm thickness, mounted on slides, heated at 60˚C for 30-40 min, dewaxed with xylene and subjected to rehydration by immersing in graded series of alcohols (100%, 90% and 70%, respectively). The sections were dipped into haematoxylin followed by eosin stain and rinsed in water. The sections were dehydrated by immersing in increasing concentrations of alcohol (70%, 90%,and 100%). following xylene and acetone for 2 min each. The images were captured in bright-field microscope at 20X magnification (Leica DM200, USA).

For IHC, Rehydrated tissue sections (3 µm thick) were washed in 1X PBS, and then the antigen was retrieved by citric acid buffer (pH 6). Non-specific site blocking and endogenous peroxide activity blocking was done by 5% fetal bovine serum (FBS) and hydrogen peroxide, respectively. Next, sections were incubated with primary antibody overnight at 4ºC. After three washes with 1X PBS, sections were incubated with Horseradish peroxidase (HRP)-conjugated secondary antibody for 30-60 min at room temperature. Sections were washed in 1X PBS and immunoreactivity was visualized using 3,3̍-Diaminobenzidine (DAB) peroxidase substrate kit (SK-4100, Vector Laboratories, CA,USA) and haematoxylin counter stain. Images were captured at 20X magnification using bright-field microscope (Leica DM2000, USA).

**Cell culture and reagents**

Breast cancer cell lines MDA-MB-231, MCF-7, cigarette smoke transformed (MCF-10A-Tr), and colorectal cancer cell line, HCT-116 were cultured in DMEM supplemented with 10% FBS, 1.5 mM L-glutamine and 1% antibiotics (100 U/ml of penicillin, 10 mg/ml of streptomycin) in a humidified incubator in 5% CO2 at 37˚C. Chemo-resistant cell line, 5-FU-R, Sensitive (H-357) and cisplatin-resistant (CIS-R) oral cancer cell lines were maintained according to the protocol described earlier (24,43). Ficoll-400 (F4375) was obtained from Sigma-Aldrich (St. Louis, USA). Anti-Nectin-4 (#ab57873), anti-CD-44 (#ab23557), anti-Oct4 (#ab109183) and anti-Afadin (#ab90809) antibodies were purchased from Abcam (MA, USA). Anti-Akt (#9272), anti-PI3K (#4292), anti-β-catenin (#9587), anti-c-myc (#9402), anti-cyclin D1 (#2922), anti-p53 (#9282) and anti-Ang-II (#2948) antibodies were purchased from Cell Signaling Technology (MA, USA). Anti-VEGFA (MAA143HU21) and anti-Ang-I (MAA008HU21) antibodies were obtained from Cloud-Clone Corp. (TX, USA). Anti-Nanog (sc-293121), anti-ALDH1 (sc-166362) and anti-GAPDH (sc-25778) antibodies were purchased from Santa Cruz Biotechnology Inc. (CA, USA).

**Western Blot analysis**

Tumor and normal tissue samples were lysed by modified RIPA lysis buffer using tissue homogenizer. Following lysis, western blot analysis was performed as described in our earlier studies (38). Briefly, 60 µg of protein was loaded and separated by SDS-PAGE. Proteins were transferred onto PVDF membrane and probed with specific antibodies as per manufacturer’s protocol. Each blot is a representative of three independent experiments. The band intensity of each lane was measured using UVPGelDoc-It®310 and represented by numerical values above proteins band in each panel.

**Enzyme-linked immunosorbent assay (ELISA)**

Expression of Nectin-4 and other proteins were assayed in circulating tumor cells (CTCs) using indirect ELISA. The experiment was performed as per the protocol mentioned earlier (5). Briefly, the protein antigen was mixed with coupling buffer, coated onto 96-well microplates and incubated overnight at 4˚C. Then, cells in each well were washed with wash buffer followed by blocking with blocking solution (1% BSA in PBST) and incubated at room temperature for 30 min. Cells were washed with PBST and incubated with HRP conjugated secondary antibody for 1 h. After washing with PBST, substrate solution (2, 2’-azinobis-(3-ethylbenzthiazoline-6-sulphonic acid) was added and the absorbance of the colored product was measured using microplate reader at 405 nm.

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

Total cellular RNA was extracted from cell lines, frozen tumors and adjacent non-tumor tissues using TRIzol reagent (Invitrogen, CA, USA) according to the manufacturer’s instructions. The cDNA was synthesized from 1 µg of total RNA using the R2D First Strand cDNA Synthesis Kit (GCC Biotech, Kolkata, India). Amplification of a 144-base pair sequence and 266-base pair sequence analogous to *Nectin-4* and *GAPDH*, respectively, was performed with the following primers: *Nectin-4* = forward 5’-TGCTCAAGTGCCTGAGTGAA-3’ and reverse 5’-AGACGTAGATGCCGCTGTG-3’ and *GAPDH* = forward 5’-GAAGGTGAAGGAGTC-3’ and reverse 5’-GAAGATGGTGATGGGATTTC-3’. Messenger RNA (mRNA) expression of Nectin-4 was performed using Hi-G9 taq DNA polymerase. The cycling conditions were as follows: an initial denaturation step of 95˚C for 5 min, followed by 35 amplification cycles involving denaturation at 95˚C for 30 sec, annealing at 50˚C for 45 sec, extension at 72˚C for 30 sec, and final extension at 72˚C for 5 min. Expression of *GAPDH* is used as loading control to check the equal loading of sample in each lane. The fold-change in the expression of mRNA levels of *Nectin-4* is calculated with respect to *GAPDH*. The amplified product was separated on 1% agarose gel and the intensity of each sample was measured using UVP imaging system (UVP, Cambridge, UK).

**Isolation of circulating tumor cells**

Circulating tumor cells from patient’s blood was isolated as per the protocol described earlier (41). Briefly, blood was drawn into EDTA-containing tube and subjected to Ficoll (1.077 g/ml) density gradient centrifugation at 200×g for 20 min at RT (15˚C - 25˚C). The upper plasma layer was carefully removed and discarded. The mononuclear/lymphocyte cell layer at the plasma-Ficoll interface was transferred to a new tube, washed twice in 1 X PBS and re-diluted with 1 X PBS and centrifuged at 230×g for 10 min at room temperature. The supernatant was discarded and the cells were resuspended with RBC lysis buffer (NH4Cl, NaHCO3 and EDTA) and incubated for 10 min at room temperature to lyse erythrocytes. Then, diluted with 1 X PBS and centrifuged at 230×g for 10 min. The supernatant was discarded and the pellet was resuspended in 1 X PBS.

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**Supplementary Fig.1.** Fold-change expression of tumor-associated proteins in all three grades of IDC determined by western blot analysis is shown. Bar Graphs (A-G) represent the expression of Nectin-4, PI3K, Akt, NFκβ, GSK-3β, Gli-1 and β-catenin, in paired adjacent normal tissues and tumor samples of different grades from I-III, respectively. Data are the mean ± SD of three independent experiments. Statistical significance was determined by paired *t*-test (\*p<0.05), (\*\*p<0.01), (\*\*\*p<0.001).

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**Supplementary Fig. 2.** Comparison of fold-change in the expression of tumor-associated proteins among different histological grades of IDC by western blot analysis. Bar Graphs (A-C) show the expression of Nectin-4, PI3K, Akt, NFκβ, GSK-3β, Gli-1 and β-catenin, in tumor samples with different grades from I-III, respectively. Statistical significance was determined by paired *t*-test (\*p<0.05), (\*\*p<0.01), (\*\*\*p<0.001).

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**Supplementary Fig 3.** **Expression of representative markers of PI3K-Akt-NFκβ signaling pathway.** Autoradiographs show the expression level of Nectin-4, Afadin, NFκβ, Akt, p53, cyclin-D1 and c-myc in paired adjacent normal tissues and tumors of breast, colon, recurrent tongue, ovary, stomach and renal. GAPDH serves as a loading control. The numerical values above each blot as determined by densitometer represent a relative fold-change with respect to the control. Data are the mean ± SD of three independent experiments.