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Article

Clinical Significance of Nectin-4 Expression in Metastasis and Angiogenesis for Tumor Relapse

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Abstract: In the present study, we have systematically examined the clinical significance of Nectin-4 (encoded by the *PVRL-4* gene), a marker for breast cancer stem cells (CSCs), in cancer metastasis and angiogenesis using a variety of human specimens, including invasive duct carcinoma (IDC) with multiple grades, several types of primary tumors to local and distant relapses, lymph node metastases and circulating tumor cells (CTCs). Nectin-4 was overexpressed in more than 92% of samples with 65.2% Nectin-4 positive cells. The level of expression was increased with increasing tumor grade (GI-III) and size (T1-4) of IDC specimens. More induction of Nectin-4 was noted in relapsed samples from a variety of tumors (colon, tongue, liver, kidney, ovary, buccal mucosa) in comparison to primary tumors, while paired adjacent normal tissues do not express any Nectin-4. A high expression of Nectin-4 along with other representative markers in CTCs and lymph node metastasis was also observed in cancer specimens. An increased level of Nectin-4 along with representative metastatic (CD-44, Sca1, ALDH1, Nanog) and angiogenic (Ang-I, Ang-II, VEGF) markers was noted in metastatic tumors (local and distant) in comparison to primary tumors that were correlated with different grades of tumor progression. In addition, greater expression of Nectin-4 was observed in secondary tumors (distant metastasis, e.g., breast to liver or stomach to gallbladder) in comparison to primary tumors. Nectin-4 was overexpressed at all stages of metastasis and angiogenesis, thus appearing to play a major role in tumor relapse through the PI3K-Akt-NFκβ pathway.

Keywords: Nectin-4; metastasis; angiogenesis; circulating tumor cells; cancer relapse

1. Introduction

Metastasis and angiogenesis are the two main obstacles for treatment of cancers. Metastasis is a complex process by which malignant cancer cells spread into other parts of the body and form secondary tumors that become more resistant to chemotherapeutic drugs (1,2). Although surgical resection and adjuvant therapy are capable of curing well-confined primary tumors, metastatic cancer is largely incurable because of its systemic spread and the resistance of disseminated tumor cells to current therapies (3). One of the major causes of tumor relapse and resistance to therapy can be attributed to the presence of cancer stem cells (CSCs). These are a small population of cells residing within the bulk of tumors. CSCs are involved in the phenotypic epithelial to mesenchymal transition (EMT) and mesenchymal to epithelial transition (MET), exhibit self-renewal ability, and

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possess high DNA repair and drug-efflux capabilities (4-9). Cancer metastasis initiates with the invasion of tumor cells through the wall of blood/lymph vessels with subsequent spread to distant sites via formation of numerous new vascular networks, a process known as angiogenesis (10). Tumor-secreted angiogenic factors activate nearby endothelial cells to release certain proteases which degrade the basement membrane and allow endothelial cells to migrate, proliferate and form new vessels (11). The angiogenic switch is a critical and important step in the transition of a dormant tumor to a malignant phenotype (12). Under hypoxic conditions, tumor cells promote the formation of new blood vessels by secreting various pro-angiogenic growth factors and by changing the expression of cell adhesion molecules, such as matrix metalloproteinases (MMPs) that facilitate cancer invasion and migration (13,14).

Previously, several markers of metastasis (e.g., CD44, Sca1, ALDH1, CXCR4, Oct 4 and c-Met) (15,16) and angiogenesis (e.g., VEGF, TGF-β, cxcl2, Angiopoietins (Ang-I and Ang-II) and iNOS) have been reported (17). Multiple drugs have been developed to target these markers in various types of cancers (18-21). There are several Food and Drug Administration (FDA) approved breast cancer drugs (https://www.cancer.gov/about-cancer/treatment/drugs/breast) that are found to benefit patients with advanced tumors. However, the theoretical promise of anti-angiogenic agents has yet to be fully translated into beneficial medical practice. In particular, drugs either alone or in combination targeting metastatic and angiogenic processes in breast tumors are still lacking (22). The clinical failure of anti-angiogenic drugs against breast cancer could also be due to as yet undiscovered factors responsible for metastasis and angiogenesis. For example, the failure of Avastin (Bevacizumab) to inhibit VEGF-mediated angiogenesis in metastatic breast cancer suggests that other factors must also be playing vital roles for these processes (23). Thus, there is an urgent need for new drugs that can effectively target metastatic and angiogenic breast tumors.

Recently, using an *in vitro* cell culture system, we and others have shown that Nectin-4 (Uniprot accn# Q96NY8, encoded by the *PVRL-4* gene), has a potential role in drug resistance, cancer cell growth, aggressiveness and metastasis (24-27). Nectins are a family of four (Nectin-1 to 4) Ca^{2+}-independent immunoglobulin-like molecules, which contribute to cell-cell adhesion by heterophilic and homophilic interactions (28). Nectins are connected to the actin cytoskeleton through F-actin binding protein (Afadin), and thereby regulate several cellular activities ranging from movement, polarization, differentiation, during entry of viruses, and involve different cell adhesion molecules and signal transduction pathways (29). Nectin-4 expression is confined to the embryo and placenta, whereas the other three Nectins are widely expressed in normal adult tissues (28,30). Overexpression of Nectin-4 has been reported in several human cancers, including lung, ovarian, and breast (31). Further, expression of the soluble form of Nectin-4 can be a potent diagnostic marker for cancers (32-34). Nectin-4 is also overexpressed in hepatocellular carcinoma (HCC) and can be used as a prognostic marker for HCC (35).

In *in vitro* studies, Nectin-4 has been shown to promote anchorage-independent cell growth by facilitating cell-cell attachment and maintaining transformed properties of breast cancer cells (36). A higher expression of epithelial markers and lower expression of mesenchymal markers in parental cell lines as compared to the Nectin-4-knockdown cells suggests a significant role of Nectin-4 in the EMT/MET pathway (37). In previous studies, a positive correlation between Nectin-4 with Ki-67 and VEGF has been linked with tumor cell proliferation and angiogenesis (25). We have also reported that Nectin-4 promotes anchorage-dependent cell proliferation and is responsible for 5-fluorouracil (5-FU) resistance in colorectal cancer cells (24). In addition, Nectin-4 is a CSC as well as EMT marker that is induced by Wnt/β-catenin signaling via the signaling PI3K/Akt axis (27). These and other studies used *in vitro* cell culture model to describe the role of Nectin-4 in cancer progression, invasiveness, and metastasis; however, very few reports directly demonstrate the role of Nectin-4 in cancer biology in patients. In this study, we explored the role of Nectin-4 in cancer metastasis and angiogenesis using different types of patient-derived

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tumor biopsy specimens with different histological grades, benign and invasive, multiple metastases (lymph node, local and distant metastasis), as well as recurrence conditions.

2. Results

Nectin-4 expression associates with tumor invasiveness

To understand the relationship between Nectin-4 expression and the invasiveness of the tumor, we monitored the expression of Nectin-4 along with representative tumor invasiveness markers in breast tissue from patient samples using IHC analysis. For this study, we collected more than 100 invasive duct carcinoma (IDC) specimens of different histological grades (grade I-III; GI: n-22, GII: n-28 and GIII: n-50) along with their paired adjacent normal tissues. The detailed clinicopathological parameters of each patient sample and other information (e.g., age, tumor size, grade and lymph node metastasis) have been provided in Table 1. H&E staining of all IDC samples showed characteristic cancerous nature and marked differences in paired adjacent normal tissues (Fig. 1a). The IHC staining of each slide was evaluated by calculating the H-score according to the procedure described in materials and methods. The H-score of the relative expression of representative proteins in different grades was plotted in graphs on the right side of each panel of Figure 1b-h. The expression was observed in all tested markers, such as Nectin-4, PI3K, NFκβ, Akt, GSK-3β, Gli-1 and β-catenin. All samples examined showed positive staining, and a great majority overexpressed the following: Nectin-4 (92%), PI3K (82%), Akt (88%), NFkβ (66%), Gsk-3β (78%), Gli-1 (84%), and β-catenin (90%). It was also noted that the expression of each marker increased with the increasing grade (GI to G-III) of tumors. Among 100 samples, approximately 6/22 (28%) GI, 21/28 (77%) G-II, and 48/50 (92.8%) G-III, respectively, showed positive Nectin-4 staining. The mean percentage of cells showing Nectin-4 staining was 65.2%, which was correlated with tumor grade. Similar trends were also noted in other markers, such as PI3K, Akt, NFκβ, Gli-1 and β-catenin (Table 1). Thus,the expression of proteins associated with tumor aggressiveness positively correlated with grades of tumors where Nectin-4 expression was higher than other proteins (Fig. 1).

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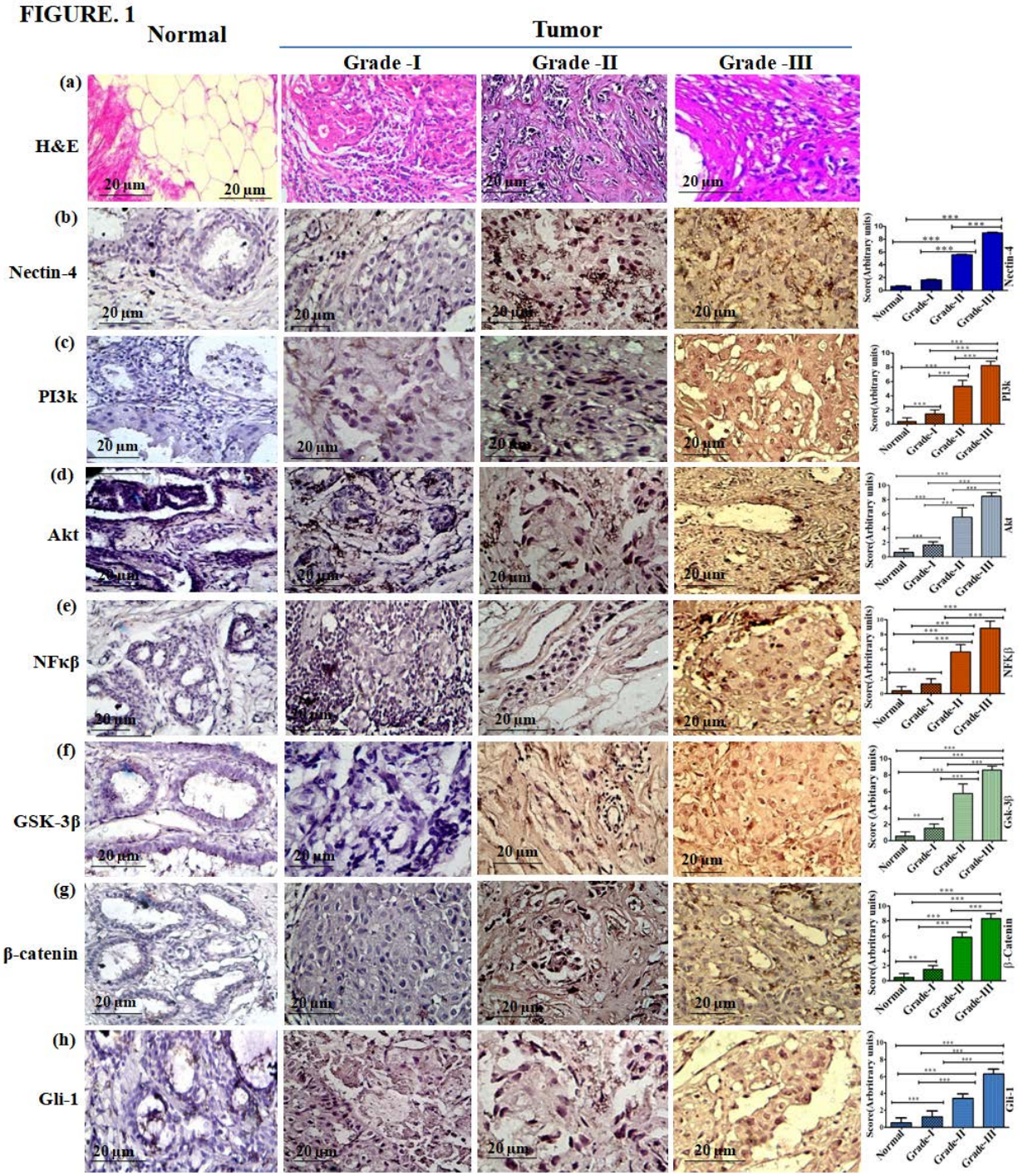

Figure 1. Expression of representative tumor invasive markers in different grades of invasive duct carcinoma (IDC) specimens. (a) Histopathologial examination of H&E stained slides of paired adjacent normal tissue and different grade (I-III) of IDC tumors. (b-h) Expression of Nectin-4, PI3K, Akt, GSK-3β, NFκβ, β-Catenin and Gli-1, respectively, by IHC analysis in paired adjacent normal tissue and different grade (I-III) of IDC tumors. The bar graphs on right side of IHC data indicate the H-score of the staining. Photographs were captured at 20X magnification under bright field microscope. Statistical significance was determined by paired *t*-test (*p<0.05), (**p<0.01), (***p<0.001). Scale bar represents 20 µm.

Histopathological evaluation showed that most of the patients exhibited positive Lymph node metastasis except six (Lymph node negative (N_0)). Immunostaining revealed positive staining (mean nuclear/cytoplasmic stain) for Nectin-4 (85.3%), PI3K (75.5%), Akt (74.40%), GSK-3β (75.7%), NFκβ (88.03%), β-catenin (86.53%), and Gli-1 (85.83%) of cells in lymph node positive samples (Table 1).

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Further, tumor size was also correlated with Nectin-4 expression as well as other above- mentioned tumor- associated markers. For the samples from the few patients in this study with T4 type tumor, all expressed Nectin-4 and the above mentioned tumor associated markers. Samples with T3-type tumors revealed Nectin-4 expression in 69.3% of IDCs with mean nuclear staining of 95%. T2- and T1-types displayed Nectin-4 expression in 76.22% and 24.5% of IDCs with a mean nuclear staining of 40.1% and 21.61%, respectively. Thus, the above data showed that Nectin-4 expression increased not only with increasing grade but also tumor size (Table 1).

Table 1. Expression of Nectin-4, PI3K, Akt, NFκβ, GSK-3β, β-catenin and Gli-1 in invasive breast carcinoma specimens and their correlation with clinicopathological parameters.

Histopatho-logical parameters		No. of specimens	Nectin-4 positive %(*)	PI3K positive %(*)	Akt positive %(*)	NFκβ positive %(*)	GSK-3β positive %(*)	β-Catenin positive %(*)	Gli-1 positive %(*)
Age	<50	70	60(73.66±28)	66(57.92±25.3)	60(70±28.4)	56(66±28.45)	58(70.92±28.4)	64(69.37±28.4)	62(68.87±28.45)
	>50	30	22(92.7±28.45)	14(85±10.69)	18(81.66±28.45)	14(86.42±28.45)	26(83.46±25.45)	28(82.14±28.45)	16(88.75±28.45)
Histological Type	Ductal	100	92(65.21±24.84)	82(57.43±25.54)	88(57.04±24.36)	66(64.09±23.40)	78(58.58±21.77)	90(55.66±23.98)	84(54.28±25.10)
Tumor (T)-classification	T1 (<2)	16	4(20±5)	5(17±7.48)	2(15)	4(10±5)	6(11.66±4.7)	8(12.5±4.33)	6(11±3.3)
	T2 (2-5)	18	14(40.7±4.94)	12(35±8.16)	14(35±7.55)	16(41.25±4.84)	14(48.9±6.99)	12(45)	14(40.71±7.28)
	T3 (>5)	64	60(95)	54(93.93±3.093)	50(93.21±3.04)	48(89.17±7.59)	44(93.5±3.4)	42(93.5±3.4)	42(92.6±5.25)
	T4	2	2(95)	2(95)	2(95)	0	2(95)	0	2(95)
Grading of invasive duct carcinoma (IDC)	G-I	22	6(15)	4(15)	8(12.5±4.33)	6(15)	10(13.75±6.28)	4(15)	6(11.66±4.71)
	G-II	28	22(41.36±6.42)	24(38.33±7.45)	26(38.84±6.24)	24(38.33±4.7)	20(42±4.58)	26(45)	24(40±6.45)
	G-III	50	48(92.45±4.33)	40(89.5±7.39)	42(77.33±8.10)	40(90±7.63)	42(89.28±7.28)	46(88.47±7.58)	44(88.18±8.19)
Lymph node metastasis	Negative-0	6	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)	0(0)
	Positive-N1(1-3)	12	8	2	6	4	0	6	0
	N2(4-9)	34	30	24	32	30	22	28	30
	N3(>10)	48	48	42	46	42	43	44	42
			86(67.79±27.85)	68(62.59±29.91)	84(61.19±26.89)	76(58.94±26.61)	65(58.30±26.84)	78(60.12±29.60)	72(61.23±28.30)

* mean nuclear/cytoplasmic staining (%) ±standard deviation

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Nectin-4 promotes breast cancer progression mediated through PI3K-Akt signaling in clinical specimens

Using an *in vitro* cell culture model, we have previously demonstrated that Nectin-4 plays a role in the maintenance of self-renewal of cells through Wnt/β-catenin signaling via the PI3K-Akt axis (27). However, there is no systematic study of Nectin-4 in cancer relapse, invasiveness and angiogenesis directly with clinical samples. To address the issue, we monitored by western blotting the protein expression of Nectin-4 along with other representative cancer markers and their corresponding signaling components in different grades of IDC specimens and paired adjacent normal tissues, respectively. The expression of PI3K and Akt was increased by 4.0- and 3.0-fold in G-III, followed by 2.0-fold in G-II and 1.5-fold in G-I tumor as compared to paired adjacent normal tissues (Fig. 2A.i). The expression of NFκβ and GSK-3β was also increased by more than 4.0-fold in G-III tumor, followed by 2.5 and 2.0-fold in G-II, and more than 1.5-fold in G-I tumor, respectively (Fig. 2A.i). The expression of β-catenin and Gli-1 was increased by more than 4-fold in G-III tumor, followed by a 2.5- and 2.0-fold in G-II, and by only 1.5- and 1.2-fold in G-I tumor, respectively (Fig. 2A.i). However, the expression of Nectin-4 was 7- and 5-fold higher in G-III and G-II, respectively, as compared to G-I tumor. In stark contrast, none of the 100 paired adjacent normal samples examined showed any expression of Nectin-4 (Fig. 2A.i). Thus, these results indicate that increased Nectin-4 expression is correlated to histological grades and is overexpressed in aggressive/poorly differentiated tumors.

Next, we measured the expression of all the above-mentioned proteins in normal, primary and local relapsed tumor samples from the same patient. As shown in Figure 2A.ii, the expression of PI3K, Akt, NFκβ, GSK-3β, β-catenin, and Gli-1 was increased by 4.5 to 6.0-fold and 1.5 to 3.0-fold in recurrent and primary tumors, respectively, as compared to corresponding paired adjacent normal tissues. The expression level of Nectin-4 was increased by more than 8-fold in relapsed as compared to primary tumors and paired adjacent normal tissues (Fig. 2A.ii). The fold-change in expression of each protein in all three grades and the fold-change in expression of these proteins in different histological grades (G-I, G-II, and G-III) are shown in Supplementary Figure 1 and 2. Thus, these results suggest that Nectin-4-mediated cancer progression may utilize GSK3/β-catenin (Wnt-Tcf pathway) and Gli-1 (Hedgehog pathway) signaling cascades for cell-renewal that also requires the PI3K-Akt-NFκβ signaling cascade.

RT-PCR was employed to examine the Nectin-4 gene expression at the mRNA level in different cancer cell lines: breast (MCF-7, MDA-MB-231, and MCF-10AT), oral (H-357 and cisplatin resistance H-357 (CIS-R)), colon (HCT-116 and 5-fluorouracil resistant HCT-116 (5-FU-R)) along with multiple primary and relapsed tumor samples. As expected, there was no Nectin-4 mRNA expression in MDA-MB-231 cells which have lost the Nectin-4 gene. Elevated Nectin-4 mRNA was observed in the cigarette smoke-transformed breast epithelial cell line, MCF-10AT (Fig. 2B.i). About 2.0- and 3.5-fold higher expression of Nectin-4 mRNA was noted in MCF-7 and MCF-10AT cells, respectively, as compared to the control MDA-MB-231cells (Fig. 2B.i). Furthermore, the expression of Nectin-4 mRNA in two drug resistant cell lines, 5-FU-R and CIS-R, was 6.5-and 5.5-fold, respectively, higher than their parental control HCT-116 and H-357 cell lines, respectively (Fig. 2B.i).

Additionally, Nectin-4 mRNA levels were assessed in various recurrent tumors along with their primary cancer and normal tissue counterparts, such as breast, tongue, renal, buccal mucosa, colon, ovary, and inguinal node. Interestingly, an increase of more than 6.0-fold was detected for Nectin-4 mRNA in all of the recurrent tumors compared to their primary tumors, with no expression in paired adjacent normal tissues (Fig. 2B.ii). These results thus imply that Nectin-4 may be responsible for drug resistance and relapse of tumors.

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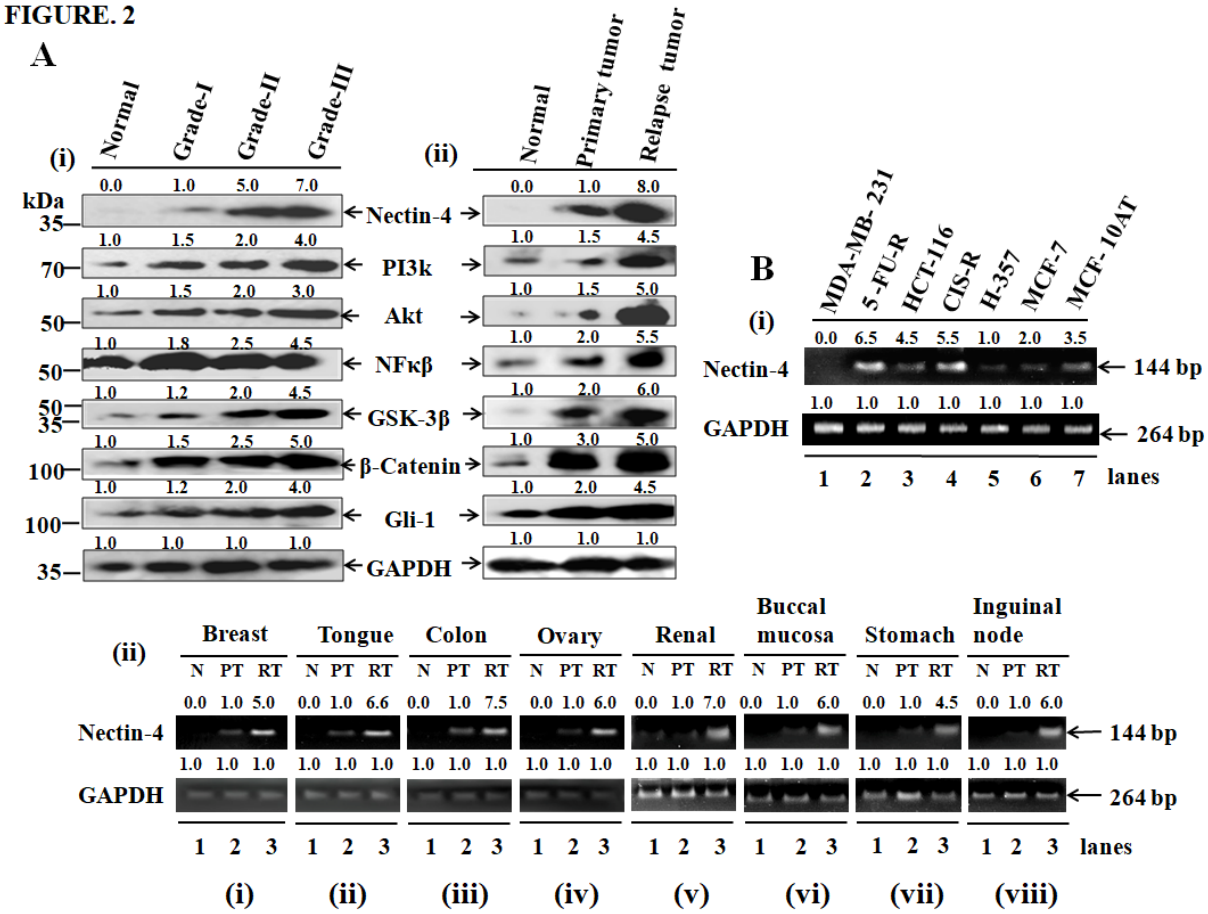

Figure 2. Nectin-4 is linked with tumor progression. Relative expression of representative tumor proliferation markers in IDC and relapsed specimens were measured by western blotting. Expression of Nectin-4, PI3K, Akt, GSK-3β, NFκβ, β-catenin and Gli-1 is shown in: **A,** (i) normal tissue and different grade (I-III) tumor specimens, and (ii) normal tissue, primary and relapsed tumor specimens. GAPDH in each set of western blot is used for loading control. The numerical values above each blot, as determined by densitometer, represent a relative fold-change with respect to the control. **B,** Overexpression of *Nectin-4* mRNA in recurrent tumors: (i) shows the *Nectin-4* mRNA levels in drug-sensitive and in resistant (5-FU-R and CIS-R) breast cancer cell lines. (ii) Shows the comparison of *Nectin-4* mRNA levels in specimens of breast, tongue, colon, renal, buccal mucosa, stomach and inguinal node with paired adjacent normal tissue and primary and relapsed tumors. The expression of *GAPDH* mRNA served as an internal control. Results are expressed as fold-change with respect to lower Nectin-4 expressing cell lines and tumor tissues and are represented in numerical values above each blot. Data are the mean ± S.D. of three independent experiments.

Increased level of Nectin-4 expression is associated with cancer recurrence

From the above results, it appears that Nectin-4 may have a potential role for breast cancer progression and relapse. Next, we extended our studies to other cancers, including ovary, buccal mucosa, tongue, sub-mandibular mass, tonsillary fossa and larynx. We took three specimens of each patient's paired adjacent normal tissue, primary, and relapsed tumors. H&E staining clearly distinguished the normal and tumor samples (Fig. 3A and B). Increased Nectin-4 expression was observed in recurrent tumors as compared to the primary tumors and their paired adjacent normal tissues (Fig. 3A and B). The H-scoring of Nectin-4 expression in histological sections of different type of cancers is also represented as bar graphs below each panel in Fig. 3A and B. All cancer samples showed elevated Nectin-4 expression, which was approximately 3-fold higher in all types of relapsed tumors compared to their primary tumors. However, we did not observe Nectin-4 expression in paired adjacent normal samples of the 12 relapsed specimens (Fig. 3A and B).

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FIGURE. 3

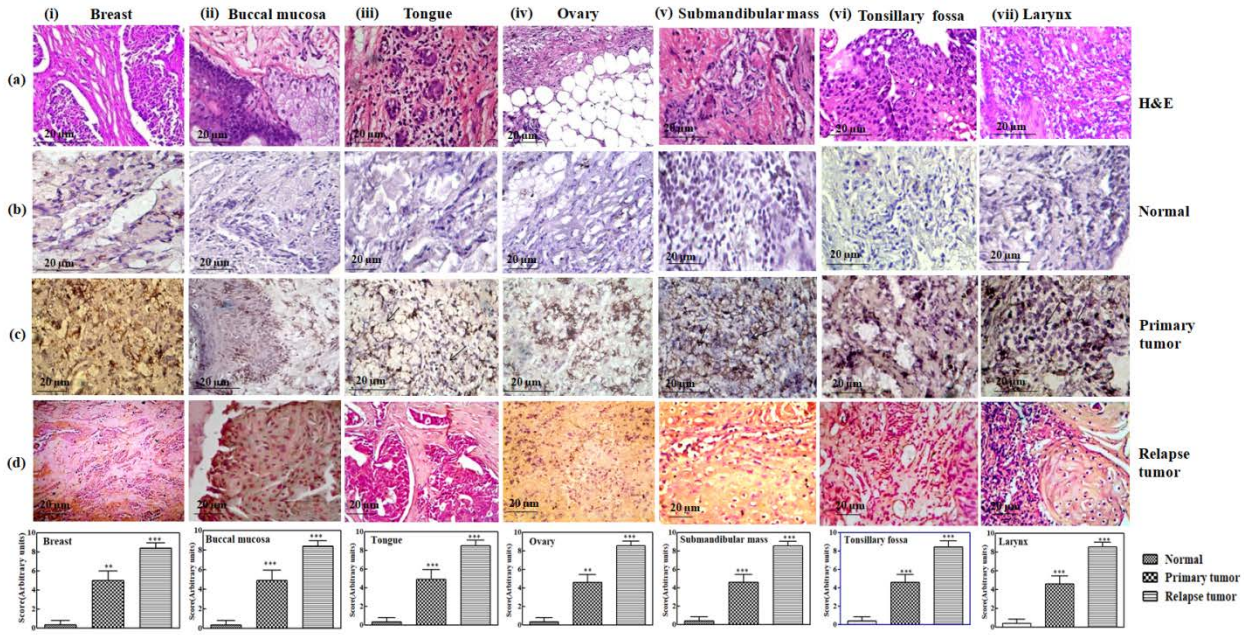

Figure 3. Overexpression of Nectin-4 in different recurrent tumors. Columns (i-vii) show the expression of Nectin-4 in breast, buccal mucosa, tongue, ovary, submandibular mass, tonsillary fossa and larynx cancer. Row a shows histopathology of H&E staining of different type of recurrent tumors. Rows b, c and d are normal tissue, primary and relapse tumor, respectively. The bar graph below each panel shows the H-score of IHC slides. The photographs were captured by bright field microscopy at 20X magnification. Statistical significance was determined by paired *t*-test (*p<0.05), (**p<0.01), (***p<0.001). Scale bar represents 20 μm.

Involvement of Nectin-4 in metastasis and angiogenesis

Though the angiopoietins Ang-I and Ang-II both are expressed at appreciable levels in the majority of breast cancers, Ang-II shows explicit association with tumor aggressiveness. Thus, Ang-I and Ang-II can be effective predictors for recurrence of both node-positive and node-negative breast cancers (42). Despite the fact that lymph node status is considered an important prognostic indicator of cancer metastasis and/or advanced staging of cancer, most instances of lymph node-negative breast cancer still relapse. Our IHC data indicate that the primary tumor and the axillary lymph nodes (ALN) express Nectin-4 in addition to the angiogenic elements, Ang-I and Ang-II (Fig. 4A). The expression of Ang-I was lower in primary tumors and axillary lymph nodes as compared to the expression of Ang-II (Fig. 4A). More than 60% and 30-40% of cells showed Ang-II and Ang-I positive cells in axillary lymph nodes, respectively. The primary tumor tissue displayed 50% of Ang-II and 30% of Ang-I positive cells. The paired adjacent non-tumor tissue showed very little expression of Ang-II and Ang-I. Taken together, since Nectin-4 is expressed in both metastatic and angiogenic tumors, it can be a bonafide marker for metastasis and angiogenesis in patients with IDC, while Ang-I and Ang-II status is likely a marker of angiogenesis only.

To explore the role of Nectin-4 in migration and proliferation of cancer cells, the expression of Afadin, NFκβ and Akt were evaluated in paired adjacent normal tissues, primary tumor and axillary lymph node of breast cancer (IDC) specimens by western blotting. Afadin binds to Nectins via actin filaments leading to cell-cell adhesion and tight junction formation (43). In these experiments, we observed a more than 3.0-fold decrease of Afadin in ALNs as compared to the primary tumors (Fig. 4B). This indicates the loss of cell-cell adhesion junctions, thereby regulates EMT in metastatic tumors that overexpress Nectin-4 (6.0-fold activation as compared to the primary tumor) (Fig. 4B). A more than a 4.0-fold increased expression of the transcription factor NFκβ, and serine/threonine kinase

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Akt, which plays a critical role in preventing apoptosis and enhancing cell survival, was found in ALNs as compared to their primary tumors (Fig. 4B).

FIGURE. 4

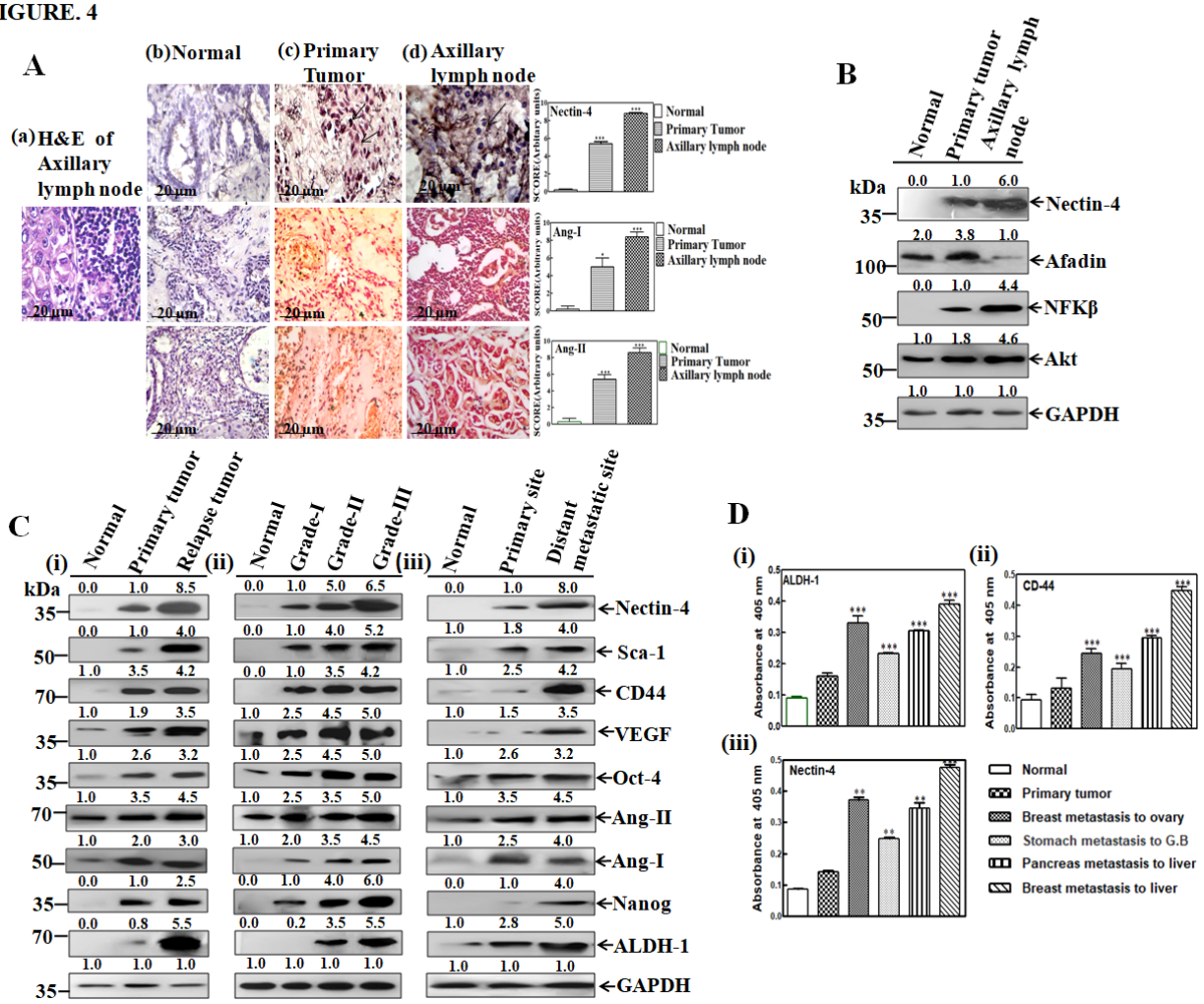

Figure 4. Role of Nectin-4 in angiogenesis and metastasis. Overexpression of Nectin-4 in axillary lymph nodes. **A,** (a) Histopathology of H&E staining of axillary lymph nodes. Rows (b-d) show the expression of Nectin-4, Ang-I and Ang-II by IHC analysis in normal (left panel), primary tumors (middle panel) and axillary lymph nodes (right panel). The bar graphs on the right side of IHC data indicate the H-score of IHC slides. The photographs were taken by bright field microscopy at 20X magnification. **B,** Western blot analysis showing the expression of Nectin-4, Afadin, NFκβ and Akt. **C,** Expression of representative metastasis and angiogenesis markers. Expression of metastasis markers (Sca-I, CD 44, Oct 4, Nanog and ALDH1) and angiogenic markers (VEGFA, Ang-I and Ang-II) is shown in panels: (i) paired adjacent normal tissue, primary and relapsed tumors, (ii) paired adjacent normal tissue and different histological grades (grade I-III), and (iii) paired adjacent normal tissue, primary and distant relapsed tumors. GAPDH served as the loading control. The numerical values above each blot as determined by densitometry represent fold-change with respect to the control. **D**, Nectin-4 expression in circulating tumor cells (CTCs). ELISA showing the expressions of CSC markers, (i) ALDH1 (ii) CD44 and (iii) Nectin-4 with CTCs from patients with different metastatic cancers. Samples include normal blood, primary cancer without metastasis, breast metastasis to ovary, breast metastasis to liver, stomach metastasis to gall bladder, and pancreas metastasis to liver. 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). Scale bar represents 20 µm.

Since our previously published *in vitro* data suggested that Nectin-4 can be a marker for breast CSCs and plays a major role in metastasis (27), in this study we examined whether this also holds true in clinical samples. For these experiments, we obtained samples from different grades (GI-III) of local as well as distant relapse sites, to measure the expression of representative metastatic and angiogenic marker proteins. The expression of CSC markers (Sca-I, CD 44, Oct-4, Nanog and ALDH-1) and angiogenic markers

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(VEGFA, Ang-I, and Ang-II) in relapsed tumors were greater than the primary tumors and the paired adjacent normal tissues (Fig. 4C.i). The expression of Sca-I, Nanog and ALDH-1 was increased by more than 4.0-fold in relapsed samples as compared to the primary tumors without showing any expression in normal tissues. The expression of CD44 and Oct-4 was increased by 4.2- and 3.2-fold in relapsed tumors, followed by primary tumors that showed activation by 3.5- and 2.6-fold, respectively, as compared to the paired adjacent normal tissues. The expression of the above proteins in normal samples was very low or negligible (Fig. 4C.i). VEGF and Angiopoietins (Ang-I and Ang-II) showed associated expression. Elevated expression of Ang-II and VEGF (3.5- and 4.5-fold, respectively) and low expression of Ang-I was observed in relapsed tumors with a very low expression in primary tumors (Fig.4C.i). Interestingly, the expression of Nectin-4 was 8.5-fold higher in relapsed tumors as compared to primary tumors. The paired adjacent normal tissues showed no expression of Nectin-4 (Fig. 4C.i). Increased expression of metastatic (Sca-I, CD 44, Oct-4, Nanog and ALDH-1) and angiogenic (VEGFA, Ang-I and Ang-II) markers was markedly noticed in the most aggressive grade, G-III, with decreased expression in G-II, followed by G-I (Fig. 4C.ii). The expression of Nectin-4 was more than 6.0-fold higher in G-III, followed by 5-fold in G-II, only 1.0-fold in G-I in IDC samples, and no expression in paired adjacent normal tissues (Fig. 4C.ii).

To further strengthen and ascertain the metastatic and angiogenic potential of Nectin-4 in these pathways, we measured the expression of the above proteins in distant relapsed samples (DRS) and their primary tumors by western blotting. Similar to the above data, expression of stemness markers (Sca-I, CD 44, Oct-4, Nanog and ALDH-1) were increased by 4.0-, 4.2-, 3.2-, 4.0- and 5.0-fold, respectively, in DRS and elevated up to 1.8-, 2.5-, 2.6-,1.0- and 2.8-fold in primary tumors as compared to the paired adjacent normal tissues (Fig. 4C.iii). Angiogenic markers VEGF and Ang-II were also increased in DRS (Fig. 4C.iii). Interestingly, the expression of Nectin-4 was 8.0-fold higher in DRS compared to primary tumor samples (Fig. 4C.iii). Taken together, these and the above results suggest that Nectin-4 may associate with metastatic and angiogenic pathways in breast cancer progression.

CTCs are the tumor cells released in the bloodstream, circulate through the body, and serve a pivotal role in metastasis. A correlation of EMT and CTCs for the formation of metastasis and angiogenesis has already been documented in a model system (44,45). To further understand whether Nectin-4 is also a prominent component of CTCs, we measured the expression of Nectin-4 as well as other established CSC markers, such as CD44 and ALDH-1 by ELISA, in CTCs isolated from blood samples from patients with different primary and metastatic cancers. An approximately 4.5- and 3.8-fold increase in ALDH-1 expression was noted in breast cancers that had metastasized to the liver and ovary, respectively (Fig. 4D.i). Further, a 3.0- and 2.5-fold elevation of ALDH1 expression was observed when a pancreatic tumor had metastasized to the liver, and a stomach tumor had metastasized to gallbladder (Fig. 4D.i). Similar trends were also found in the expression of CD44. Elevated expression of 4.5-, 2.9-, 3.0- and 2.0-fold induction of CD44 expression was noted in case of a breast tumor metastasis to liver (4.5-fold), a pancreatic tumor metastasis to liver (2.9), a breast tumor metastasis to ovary (3.0) and a stomach tumor metastasis to gallbladder (2.0) (Fig.4D.ii). Interestingly, expression of Nectin-4 was 6.0- and 3.8-fold higher in breast cancers that had metastasized to liver and ovary, respectively, as compared to paired adjacent normal tissue. There was a 3.5- and 2.5-fold elevation of Nectin-4 expression when a pancreatic tumor had metastasized to liver and a stomach tumor had metastasized to gall bladder, respectively (Fig. 4D.iii). Thus, the expression of Nectin-4 was significantly higher in CTCs of metastatic cancers than the expression of ALDH-1 and CD44.

Using cell line model systems, we have previously shown that Nectin-4 physically interacts with the adaptor protein Afadin and increases cancer cell growth through PI3K-Akt-NFκβ signaling (27). To determine whether the same pathway is activated in different clinical cancer types with elevated Nectin-4 expression, we measured the expression of

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the major components of this signaling cascade by western blotting (Supplementary Fig.3). In agreement with our *in vitro* studies (27), the elevated/activated status of these major components suggests that Nectin-4 may cause cancer cell proliferation through PI3K-Akt-NFκβ signaling.

3. Discussions

Cancer cells follow a series of complex, sequential steps during metastasis to distant organs, which are mediated by selected genes of (46): (i) Invasion and migration – Metastasis begins when some cancer cells from primary tumors undergo an EMT that is controlled by genes including *Twist*, *Slug*, *Snail and Zeb1*. The loss of E-cadherins and gain of N-cadherins or vimentin, a transition from $CD44^{-}/CD24^{+}$ to $CD44^{+}/CD24^{-}$, and expression of matrix metalloproteinases (MMPs) are the major phenomena of EMT. (ii) Circulation – the metastatic cells transit in the blood stream as circulating tumor cells (CTCs) and then attach to distant organs. These cells undergo EMT to MET transition with high heterogeneity in their morphological and phenotypic profile and grow as secondary tumors. (iii) Extravasations – the increased expression of *Ereg*, *MMP-1*, *MMP-2* and *Cox-2* genes facilitate the disruption of vascular junctions leading cancer cells to invade distant organs. (iv) Survival – the disseminated tumor cells (DTCs) colonize at secondary sites by inducing the expression of certain cell survival genes, such as *Cscl12 and Igf1*. (v) Outgrowth –the surviving cancer cells modify the distant stroma and trigger intrinsic oncogenic signaling pathways for proliferation by triggering the expression of additional genes such as *Pthrp*, *Jagg1*, *IL-8*, *IL-11*, *Cxcl1*, *Wnt* and *Notch*. The risk of metastasis development increases with lymph node metastasis, large size of primary tumor, and loss of histopathological differentiation (grade), which are well known to be cancer prognostic markers (47). In the present study, we have for the first time systematically examined the role of the cell-cell adhesion molecule Nectin-4 in clinical samples of different types of cancers at different stages of progression, local and distant metastasis, lymph nodes and CTCs.

Using an *in vitro* model system, we and others have demonstrated the role of Nectin-4 in cancer cell proliferation, drug resistance and EMT/MET transition (24,25,27,37). Although there are few studies describing the role of Nectin-4 in cancer invasiveness and metastasis in a specific type of breast cancer patients (28,35), its mechanistic role in tumor progression, metastasis, and angiogenesis with clinical samples has been lacking. Our present study has carefully evaluated the Nectin-4 expression in a cohort of breast cancer samples including their primary and relapsed samples (both local relapse and distant relapse).

We used more than 100 IDC specimens of different grades (Grade I-III) with the corresponding relapsed samples to measured Nectin-4 expression along with other representative markers for cell aggressiveness. Nectin-4 was overexpressed in more than 92% of tumor samples. In 65.2% of Nectin-4 positive tumors, the expression was increased with increasing grade (from I-III) of tumor progression. We observed a maximum expression of Nectin-4 in relapsed samples, with no expression in paired adjacent normal tissues from the same patient. Nectin-4 expression also positively correlated with tumor grade, tumor size, and lymph node status. This percentage of Nectin-4 expression is within the range of previously published studies on breast carcinoma (31,32). These findings demonstrate that Nectin-4 overexpression is associated with more aggressive clinical behaviour and presents a worse prognosis for patients. Our studies further suggest a link of Nectin-4 expression with the expression of other aggressive (Akt, PI3K, NFκβ, and GSK-3β) and CSC representative (β-catenin and HH-Gli-1) markers that were increased with increased tumor grades and in relapsed tumors. Most likely, the role of Nectin-4 in tumor aggressiveness is dependent upon Wnt-Tcf-signaling of CSC self-renewal, which is supported by our earlier *in vitro* studies (24).

To further validate the role of Nectin-4 beyond breast cancer progression, we measured Nectin-4 expression in other tumor types with primary and relapsed clinical specimens, such as ovary, tongue, colon and renal. The highest Nectin-4 expression was always

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observed in relapsed samples, whereas a moderate level in primary and no expression in normal samples support the hypothesis that Nectin-4 contributes to cancer progression. The elevated expression of Nectin-4 in association with the elevated expression of representative metastatic (CD44, Oct-4, Nanog, and ALDH1) and angiogenic (Ang-1, Ang-II, and VEGF) markers in tumor samples with Grade-I to Grade-III, relapsed as well as distant metastases, suggests that Nectin-4 also contributes to metastasis and angiogenesis. Increased Nectin-4 in association with elevated VEGF, Ang-I, and Ang-II in lymph node samples also supports the idea that Nectin-4 contributes to metastasis and angiogenesis.

The increased Nectin-4 mRNA in several cancer cell lines, including drug resistant 5-FU-R and CIS-R cells, and in relapsed tumor samples when compared to primary tumors, and no expression in paired adjacent normal samples, provides further evidence concerning the contribution of Nectin-4 to tumor progression and metastasis. Next, our findings of elevated Nectin-4 expression, along with other CTC markers from the blood samples from patients with metastases, strongly suggest that Nectin-4 expression is associated with metastasis. Further, observations of the associated overexpression of PI3K-Akt-NFκβ pathway proteins in the wide tumor types examined in this study supports our *in vitro* data (24) that Nectin-4 is associated with oncogenic Akt-PI3K-NFκβ signaling during tumor progression and metastasis.

4. Materials and Methods

Tissue specimens

Surgically resected specimens, both untreated primary breast carcinomas and paired adjacent normal breast tissues, were collected after modified radical mastectomy (MRM) from breast cancer patients enrolled in the outpatient department of Oncosurgery, Acharya Harihar Regional Cancer Centre, Cuttack, Odisha, India, after the approval of Institutional Ethics Committee (Ethical clearance approval Regd. # ECR/297/Inst/OR/2013). The age of the patients ranged from 28-85 years with a median range of 56 years. All the patients included in the study had invasive duct carcinoma (IDC) and their clinicopathological parameters are summarized in Table 1. In addition, carcinoma and adjacent normal tissues were also collected from patients with different types of cancers, such as oral, ovary, stomach, renal and colon immediately after surgery. Tumor differentiation was classified according to the Scarf Bloom-Richardson grading system (BR-score). The pathological tumor staging was evaluated by the 6th edition of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer. Both Loco-regional and distant relapses were determined by radio-imaging and biopsy. In addition, blood samples were collected in EDTA vials from patients with or without metastasis for isolating circulating tumor cells.

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

H&E staining and IHC were performed as described in our previous studies (38). The full description of methods used for H&E and IHC are provided in the supplementary file.

Evaluation of immunostaining

For evaluation of immunostaining, a semiquantitative assessment of both the intensity of staining and the percentage of positively stained cells was used. The percentage of stained cells was classified as 0 (0%-10%), 1 (11%-30%), 2 (31%-50%), and 3 (51%-100%). Staining intensity was defined as 0 (-/negative), 1 (+/weak), 2 (++/moderate) and 3 (+++/strong). Total immunoreactivity score (H-score) was the product of the staining percentage and intensity scores. A final total score $\leq$3 was regarded as low expression, while a final total score >3 was regarded as high expression (39). All tissue sections were analyzed by two independent experienced pathologists who were blinded to both the clinicopathological data and results of each other.

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Western Blot analysis

Tumor and normal tissue samples were lysed by modified RIPA lysis buffer using a tissue homogenizer. Following lysis, western blot analysis was performed as described in our earlier studies (41). A brief description of the method is provided in the supplementary file.

Reverse transcriptase polymerase chain reaction (RT-PCR)

The RT-PCR of Nectin-4 and GAPDH was carried out in sample and cells according to the protocol described in the supplementary file.

Cell culture

Breast cancer (MDA-MB-231, MCF-7, cigarette smoke transformed (MCF-10A-Tr), colorectal cancer (HCT-116), Chemo-resistant (5-FU-R, CIS-R) and chemo-sensitive (H-357) cancer cell lines were maintained according to the protocol described earlier (24, 40). Details of cell culture reagents and sources of antibodies are provided in the supplementary file.

Isolation of circulating tumor cells

Circulating tumor cells (CTCs) from patients' blood were isolated as per the protocol described earlier (41). Detail isolation of CTCs is described in the supplementary file.

Enzyme-linked immunosorbent assay (ELISA)

Expression of Nectin-4 and other proteins were assayed in CTCs using indirect ELISA. The experiment was performed as per the protocol mentioned earlier (5). The detailed method is described in the supplementary file.

Statistical analysis.

Correlation between markers and clinicopathological parameters was determined using Chi-square analysis. The statistical significance was calculated using GraphPad Prism version 5 Software (La Jolla, CA, USA). The results were represented as a mean ± SD of three independent experiments. Analysis of data was done by two-way ANOVA followed by a comparison between multiple data using Bonferroni's multiple comparison test. The statistical significance is represented as *$p< 0.05$, **$p< 0.005$ and ***$p<0.001$.

5. Conclusions:

In conclusion, Nectin-4 is predominantly expressed in tumors with the most aggressive histopathological grades and in relapsed tumors. The association of Nectin-4 with aggressive phenotypes and recurrent tumors suggest that Nectin-4 contributes to both metastasis and angiogenesis and does so by modulating key factors of their major signaling pathways. Finally, our findings in CTCs suggest that Nectin-4 contributes to EMT/MET transitions in clinical blood samples. Our data suggest that Nectin-4 is associated with all later and lethal stages of tumor progression including metastasis, angiogenesis, and relapse (Fig.5). Thus, the Nectin-4 expression should be used as a predictive marker for cancer diagnosis and can be a target for the development of targeted therapeutics.

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FIGURE .5

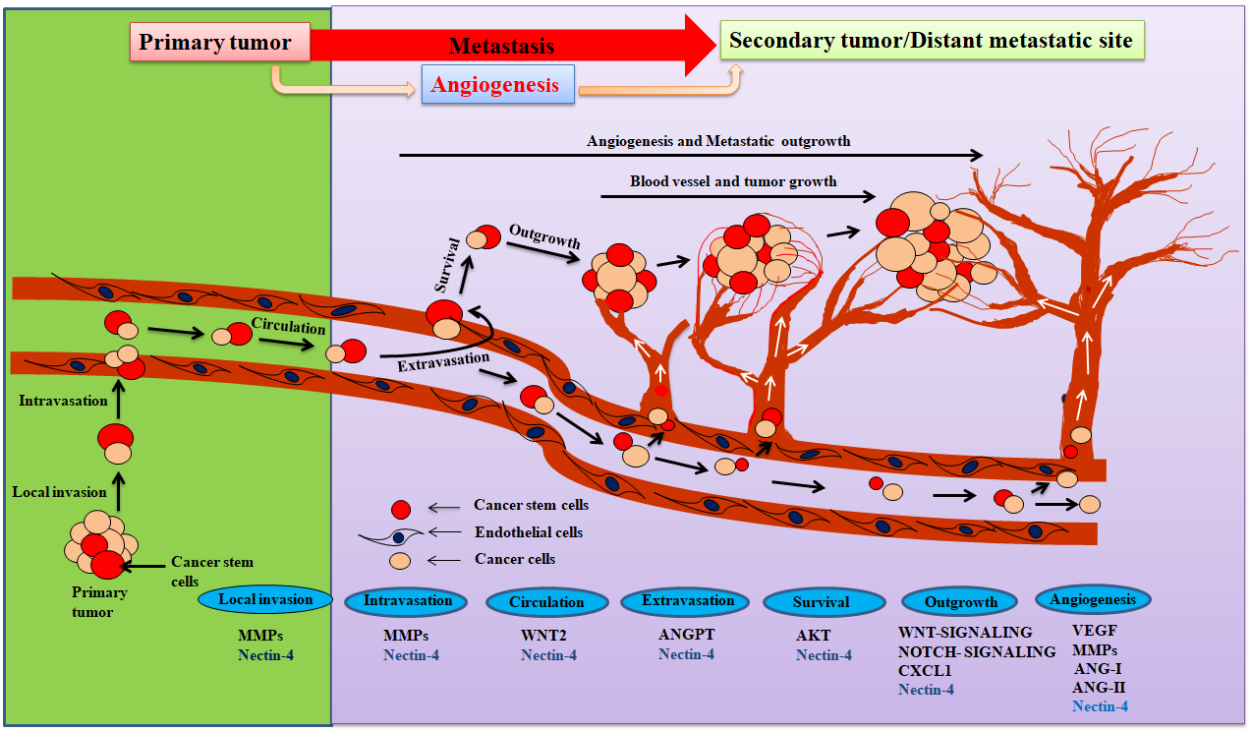

Figure 5. Schematic representation of metastasis and angiogenesis processes. The metastatic cascade is depicted starting from the primary tumor site to distant sites via (i) local invasion, (ii) intravasation, (iii) circulation, (iv) extravasation, (v) survival, (vi) outgrowth, and finally (vii) angiogenesis.

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Author contributions: CS performed the majority of the experiments. KG provided samples, NR helped to perform pathological studies. DN, RP, SC, and SM performed some of the experiments and statistical analysis. CNK conceived the idea, planned the experiments and wrote the MS. SN and MDW contributed to writing the manuscript.

Competing Interest- None

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