Clinical Significance of Wnt Acyltransferase - Porcupine in Breast Cancer

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Summary

Porcupine (Porcn) protein plays a crucial role for secretion and function of Wnt signaling molecules and thereby in Wnt-induced cell signaling, which further stimulates the expression of diverse cancer-related genes resulting in development of various cancers, including breast cancer. Hence, the present study aimed to investigate the expression of Porcn protein in patients with primary breast carcinoma and explore their relation with clinico-pathological parameters as well as the prognostic significance. The study comprised of 160 breast cancer patients who underwent surgery as the primary treatment. The protein expression of Porcn was detected by immunohistochemical staining and analyzed using H-score method. Statistical analysis was carried out using SPSS and $p \leq 0.05$ were considered significant. Porcn protein was detected in 96% (153/160) of breast cancer patients with an over expression observed in 46% (73/160) of breast cancer patients. The correlation of Porcn protein with traditional clinico-pathological parameters of breast cancer revealed that the expression was significantly higher in Her2 negative patients (54%, 49/91) as compared to Her2 positive patients (35%, 24/69; $\chi^2=5.749$, r=-0.190, p=0.016); while a trend of higher incidence was observed in patients with T1+T2 tumor size (48%, 65/134) as compared to those with T3+T4 tumor size (31%, 8/26; $\chi^2=2.762$, r=-0.131, p=0.098). However, no other significant associations were observed with rest of the breast cancer characteristics. In addition, Porcn protein failed to emerge as a significant prognosticator in breast cancer patients. Porcn protein was detected in substantial number of breast cancer patients indicating its role in malignant development. However, further studies in more number of patients are warranted for a conclusive finding.

Keywords: Wnt signaling, immunohistochemistry, breast cancer

Introduction

Secreted Wnt proteins activate signal transduction pathways which are crucial in regulating a multitude of developmental and homeostatic processes in embryos and in adults.[1,2] Wnt proteins signal through both β-catenin dependent and β-catenin independent pathways. Aberrant activation of Wnt signaling is proposed to be causal in a subset of cancers due to over expression in either upstream or downstream components.[3, 4] Thus, Wnt driven cancers can be targeted at several steps in the pathway.[5,6] One approach is to target the secretion of all Wnts by inhibiting the enzymatic activity of a multi-pass integral membrane-bound O-acyl transferase (MBOAT) Porcupine (Porcn). Porcn is essential for post-translational modification of all Wnt proteins to enable their transport, secretion and activity.[7]

Porcn catalyzes the acylation of the serine residue of Wnt required for its anchoring function in cellular membranes by inserting into the lipid bilayer. Secondly, Porcn also catalyzes palmitoylation of cysteine residue of Wnts necessary for ability of Wnts to interact with Fzd or other receptors.[7,8] Thus the post-translational lipid-modification is essentially required for trafficking through the intracellular secretory pathway, release from cell membranes and the extracellular transport.[9] Moreover, the dual lipidation of Wnts by Porcn contribute to its hydrophobic nature and could be addressed as lipidation for signaling activity.[10,11] Hence, Porcn is required for the secretion of functional Wnt in diverse organisms.

However, data regarding Porcn activity is conflicting. In vivo studies have shown that inhibition of Porcn leads to developmental disorders, most notably Goltz Syndrome which causes focal dermal hypoplasia.[12] Conversely, overactive Porcn results in cancerous cell growth.[13] Further, inhibition of Porcn has been found to be an effective strategy for broadly suppressing Wnt signaling and thus hold potential in regenerative medicine and anticancer applications.[14] Moreover, apart from Porcn mRNA studies, there is a single study by Bonne et al that has reported Porcn protein expression by immunohistochemistry (IHC) in ovarian cancer patients.[15] Hence, little is known with regard to Porcn protein expression by IHC in human cancers including breast cancer. Moreover, as Wnt-induced cell signaling stimulates the expression of various cancer-related genes, some gene expressions might also be regulated by the level of Porcn.[16] Therefore, to investigate the significance of Porcn in human breast cancer, current study examined the Porcn protein expression, correlated it with traditional clinico-pathological variables and analyzed its prognostic role in breast cancer patients.

Materials and Method

Patients:

A total of 160 untreated histologically confirmed breast cancer patients with Invasive Ductal Carcinoma (IDC) type registered at Gujarat Cancer & Research Institute from March 2014 to December 2015 were enrolled. The study was approved by the

Table 1: Clinico-pathological characteristics of Breast Cancer patients (N=160)

Variables	N (%)
Total patients	160 (100)
Age (years)	≤
≤50	81 (51)
>50	79 (49)
Menopausal status	
Pre-menopause	56 (35)
Post-menopause	104 (65)
Tumour size	
T1 (≤20 mm)	21 (13)
T2 (20-50 mm)	113 (71)
T3 (>50 mm)	17 (11)
T4 (Extension to chest wall and/or skin)	09 (05)
Nodal status	
Negative	65 (41)
Positive	95 (59)
TNM stage	
I	12 (08)
II	86 (54)
III	61 (38)
IV	1 (6)
Tumor grade	
Grade 1	13 (8)
Grade 2	103 (64)
Grade 3	44 (28)
ER	
Negative	72 (45)
Positive	88 (55)
PR	
Negative	100 (62)
Positive	60 (38)
Her2	
Negative	91 (57)
Positive	69 (43)
Molecular subtype	
Luminal A	52 (33)
Luminal B	36 (22)
Her2-positive	35 (22)
TNBC	37 (23)
Treatment administered	
S	11 (7)
S+CT	45 (28)
S+CT+RT	27 (17)
S+CT+HT	35 (22)
S+CT+RT+HT	42 (26)

S=Surgery; CT=Chemotherapy; RT= Radiotherapy; HT= Hormonal therapy

Institute's Ethics Committee Board and written consent forms were obtained from all the patients prior to treatment administration. Detailed clinical and pathological history of the patients [age, tumor-node-metastasis (TNM) stage, histopathological findings, ER, PR, Her2 status, treatment given, etc.] was obtained from the case files maintained at the Medical Record Department of our institute. All patients underwent surgery and adjuvant treatment decision based on molecular subtypes of breast cancer patients was done by clinicians of the institute. The clinico-pathological characteristics of the enrolled patients are enlisted in Table 1. Complete follow-up details of 69% (111/160) patients were obtained, who were included in overall survival (OS) analysis. Amongst these, 3% (2/111) patients had persistent disease and hence only 68% (109/160) patients were included for the analysis of relapse free survival (RFS).

Immunohistochemistry:

Porcn protein expression was studied immunohistochemically using formalin-fixed paraffin embedded tissue blocks retrieved from the tissue repository of our institute's Pathology Department. The blocks were cut into 4 μm sections and mounted on 3-amino propyl triethoxy silane (APES)-coated slides. The staining was performed using HRP/DAB (ABC) Detection IHC kit (Abcam, Cambridge, UK) according to manufacturer's protocol. Briefly, antigen retrieval treatment was given by heating the sections in 10 mM sodium citrate buffer (pH-6.0) in a pressure cooker. Then after, sections were incubated overnight at 40 C with rabbit polyclonal primary antibodiy for Porcn procured commercially (HPA049215, Sigma-Aldrich, USA) at a dilution of 1:100. The stained sections were mounted with DPX and observed under the light microscope. Sections with intense staining for Porcn were used as positive control, whereas negative control was obtained by omission of primary antibody.

Assessment of Porcn expression:

The stained sections were evaluated independently by semi-quantitative histoscore (H-score) method on the basis of staining intensity and percentage of positive cells. The staining intensity was scored on a scale of 0-3 where 0 indicated no staining obtained, 1+ for weakly stained cells, 2+ for moderately stained cells and 3+ for strong intense staining of the cells. The extent of staining was expressed by percentage of positive cells (0-100%) by 10% intervals. The final H-score was calculated by multiplying the staining positivity score with the staining intensity score of each section, ranging from 0 to 300. The mean H-score value of Porcn was 105 (range of 0 to 255) and this was used as a cut-off value to subgroup the patients into low (≤105 H-score) and high (>105 H-score) expression groups, respectively.

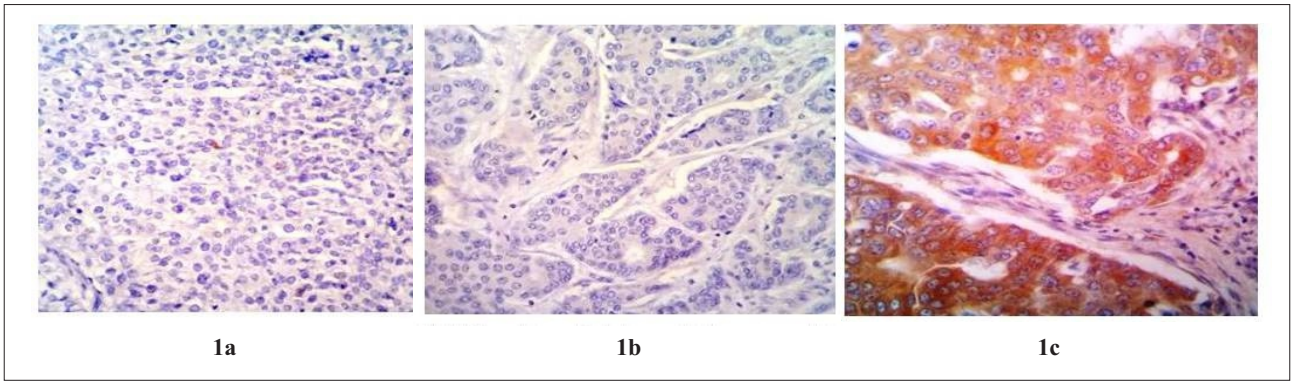

Figure 1: Representative immunohistochemical staining pattern of Porcn in Breast Cancer patients **Figure 1a:** Negative control for Porcn staining **Figure 1b:** Negative staining of Porcn protein **Figure 1c:** Cytoplasmic staining of Porcn protein in breast tumors

Statistical Analysis:

The data was analyzed statistically using SPSS Inc. version 23 software. The correlation between the expression of Porcn protein and various clinico-pathological characteristics of breast cancer patients was determined by two-tailed chi square test ($\chi 2$) and spearman's correlation. Survival analysis was performed using Kaplan-Meier survival function and the differences in survival were tested for statistical significance using log-rank statistic. $p \leq 0.05$ was considered to be statistically significant.

Results

Incidence of Porcupine protein expression in breast cancer patients

Porcn protein was detected solely in the cytoplasm of tumor cells in 96% (153/160) of breast cancer patients. Figure 1 shows the representative photomicrographs for Porcn staining. The staining intensity of 1+, 2+ and 3+ was noted in 26% (41/160), 38% (61/160) and 32% (51/160), respectively. According to the cut-off value as described previously, 54% (87/160) of patients exhibited low Porcn expression and 46% (73/160) patients exhibited high Porcn expression.

Correlation of Porcn protein with clinico-pathological features of breast cancer patients

The correlation of Porcn protein with various clinico-pathological parameters of breast cancer patients such as age, menopausal status, tumor size, nodal status, TNM stage, BR score, lymphatic permeation, vascular permeation, perineural extension and perinodal extension showed no significant association of Porcn protein expression with any of the mentioned parameters. However, when the patients were subgrouped on the basis of tumor size as T1+T2 and T3+T4, a trend of higher Porcn expression was observed in patients with T1+T2 tumor size (48%, 65/134) as compared to those with T3+T4 tumor size (31%, 8/26; χ^2=2.762, r=-0.131, p=0.098) (Table 2).

Table 2: Correlation of Porcn expression with clinico-pathological features

Characteristics	N	Porcn protein expression		χ2	R	p
		Low N (%)	High N (%)			
Age (years)						
≤50	81	41(51)	40(49)	0.934	-0.076	0.337
>50	79	46(58)	33(42)			
Menopausal status						
Pre-menopausal	56	29(52)	27(48)	0.233	-0.038	0.632
Post-menopausal	104	58(56)	46(44)			
Tumor size						
T1	21	12(57)	9(43)			
T2	113	57(50)	56(50)	4.120	2.762	-0.072
T3	17	13(76)	4(24)			
T4	09	5(56)	4(44)			
T1 + T2	134	69(52)	65(48)	-0.131	0.364	0.098
T3 + T4	26	18(69)	08(31)			
Nodal status						
Negative	65	34(52)	31(48)	0.189	-0.034	0.660
Positive	95	53(56)	42(44)			
TNM stage						
I	12	5(42)	7(58)			
II	86	45(52)	41(48)	2.296	-0.101	0.203
III	62	37(60)	25(40)			
IV	01	1(100)	00(00)			
Early (I+II)	99	51(52)	48(48)	0.856	-0.073	0.358
Advanced (III+IV)	61	36(59)	25(41)			
BR score						
Low (BR3-BR5)	13	5(38)	8(62)			
Intermediate (BR6-BR7)	103	56(54)	47(46)	1.721	-0.088	0.270
High (BR8-BR9)	44	26(59)	18(41)			
Low+Intermediate	116	61(53)	55(47)	0.544	-0.058	0.464
High	44	26(59)	18(41)			
Lymphatic permeation						
Absent	84	41(49)	43(51)	2.208	-0.117	0.139
Present	76	46(40)	30(40)			
Vascular permeation						
Absent	140	74(53)	66(47)	1.040	-0.081	0.311
Present	20	13(65)	7(35)			
Perineural invasion						
Absent	147	80(54)	67(46)	0.002	+0.003	0.968
Present	13	7(54)	6(46)			
Perinodal extension						
Absent	94	50(53)	44(47)	0.129	-0.028	0.722
Present	66	37(56)	29(44)			

Table 3: Correlation of Porcn expression with ER, PR, Her2 expression and molecular subtypes

Characteristics	N	Porcn protein expression		χ2	R	p
		Low N (%)	High N (%)			
ER						
Negative	72	43(60)	29(40)	1.509	+0.097	0.222
Positive	88	44(50)	44(50)			
PR						
Negative	100	56(56)	44(44)	0.284	+0.042	0.597
Positive	60	31(52)	29(48)			
Her2						
Negative	91	42(46)	49(54)	5.749	-0.190	0.016
Positive	69	45(65)	24(35)			
Molecular subtypes						
Luminal A	52	21(40)	31(13)	8.396	-0.117	0.140
Luminal B	36	23(64)	36(60)			
Her2 positive	35	24(69)	11(31)			
TNBC	37	19(51)	18(49)			

Table 4: Univariate survival analysis for RFS and OS in relation to Porcn protein expression in breast cancer patients

Porcn protein expression	RFS (N=109)			OS (N=111)		
	N	No recurrence N (%)	Recurrence N (%)	N	Alive N (%)	Dead N (%)
Low	64	49(77)	15(23)	65	50(77)	15(23)
High	45	35(78)	10(22)	46	40(87)	06(13)
	Log rank= 0.049, df= 1, p= 0.825			Log rank= 1.748, df=1, p= 0.186		

Correlation of Porcn protein expression with ER, PR, Her2 expression and molecular subtypes of breast cancer patients

As shown in Table 3, a significant preponderance of Porcn expression was found in Her2 negative patients (54%, 49/91) as compared to Her2 positive patients (35%, 24/69; χ^2=5.749, r=-0.190, p=0.016). However, no significant association of Porcn expression was observed with ER or PR status. In addition, the patients were subcategorized according to molecular subtypes based on ER, PR and Her2 status, where the incidence of Porcn expression was higher in Luminal A (60%, 31/52), followed by TNBC (49%, 18/37), Luminal B (36%, 13/36) and Her2 positive (31%, 11/35), although the difference was not statistically significant (χ^2=8.396, r=-0.117, p=0.140).

Survival outcome of breast cancer patients in relation to Porcn protein expression

In total patients with breast carcinoma, univariate analysis showed that Porcn protein failed to predict RFS and OS (RFS: Log rank= 0.049, df= 1, p= 0.825; OS: Log-rank = 1.748, df = 1, p= 0.186). Further, survival analysis was performed in patient's subgroups according to lymph node status, disease stage and BR score. However, Porcn protein expression did not emerge as a significant prognosticator in any of the patient subgroups (data not shown).

Discussion

An altered regulation of the proteins involved in Wnt signaling pathway is linked to the development of a wide range of human cancers, in particular breast carcinoma.[17,18] Increased activity of the Wnt/β-catenin pathway can result from upregulation at different steps in the signaling pathway: overexpression of the cell surface receptors LRP5, LRP6, and Frizzled or increased activity of the Wnt target genes survivin, cyclin D1, Axin2, and c-myc.[19-21] The endoplasmic reticulum-resident Ô-acyltransferase Porcupine is one such putative enzyme that catalyzes the lipid modification of Wnt proteins. This acylation is necessary for the Wnt secretion via Golgi as well as it contributes to the binding of Wnt to its surface receptors and, therefore, Wnt-induced cell signaling.[22-25] Hence, Porcn is a crucial component necessary for Wnt ligand transport, secretion and activity and has been identified as a potential target to inhibit Wnt/β-catenin signaling.[26] Moreover, studies by Chen et al and Mo et al have reported the role of Porcn mRNA over expression in human lung and gastric cancer, respectively.[27,28] Therefore, current study investigated Porcn protein expression, correlated it with traditional clinico-pathological variables and evaluated its prognostic role in breast cancer patients using IHC technique.

In present study, protein expression of Porcn was found to be localized in the cytoplasm of breast tumor cells. Similarly, Bonne et al also observed cytoplasmic Porcn expression in ovarian cancer samples.[15] In addition, Porcn immunoreactivity in present study was observed in 96% of patients; and on the basis of mean H-score value as cut-off, high expression of Porcn was found in 46% patients. Elevated levels of PPN/MG61 (orthologue of Porcn) mRNA expression are reported in human lung cancer cell lines as well as in human primary lung cancer tissue samples (22 out of 24), when compared to their matched normal lung tissues.[27] Similarly, the mRNA levels are reported to be over expressed in 62.5% of gastric cancer tissue samples compared with adjacent normal tissue samples. The authors also showed significant overexpression in gastric cancer cell line compared to normal tissues, implying the importance of PPN in gastric cancer.[28] Also, the mRNA expression of Porcn is found to be present in several breast cancer cell lines;[29] suggesting the potential clinical implications of Porcn in cancer cells, whereby it may promote post-translational modification of the oncogenic Wnt molecules and contributes to aberrant activation of the Wnt signaling pathway in cancer development.

Moreover, current study observed a trend of higher Porcn protein expression in patients with smaller tumor size (T1+T2) as compared to those with larger tumor size (T3+T4). However, Covey et al (2012) has shown a critical role of Porcn in cell proliferation in xenografts, where the knockdown of Porcn leads to decrease in Wnt activity and thereby development of significantly smaller and lighter tumors in mice. Additionally, high Porcn was significantly associated with Her2 negative status of the patients enrolled in present study. Moreover, no other significant associations of Porcn protein expression was observed with rest of the parameters of breast cancer patients. Further, survival analysis also showed no significant correlation of Porcn expression with RFS and OS in total patients or in any of their subgroups. However, Porcn protein is also involved in an alternative function, which is independent of acyltransferase activity and is rate-limiting for the cell proliferation and growth of transformed epithelial cells. Hence, moonlighting of Porcn performs additional Wnt-independent functions alongside their catalytic roles that promotes cancer cell proliferation and regulates expression profiles of a distinct set of genes other than Wnt pathway using the same protein domain.[29]

Moreover, there are small molecule inhibitors of Porcn being developed that are highly effective in preventing Wnt secretion and thereby therapeutically target Wnt-dependent cancer cells.[30] However, downstream activation of Wnt/β-catenin signaling is often due to APC or β-catenin mutations, which might render them insensitive to Porcn inhibition.[31] Hence, the above observations and literature survey suggests that Porcn may be a novel marker for cancer, especially human lung cancer and that post-translational modification of the Wnt signal molecules by Porcn may be important for the function of Wnt pathway in lung cancer and gastric cancer. [27,28]

Conclusion

The present study has detected the presence of Porcn protein in breast tumor cells immunohistochemically. Moreover, the protein is over expressed in substantial number of patients with a significant high expression in Her2 negative patients. Although, the association of Porcn protein with survival outcome failed to reach the level of significance, further studies are required to evaluate the prognostic role of Porcn protein expression in large number of breast cancer patients.

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" When you reach the top, keep ascending,
otherwise you start descending. "
Lincoln Patz