RESEARCH ARTICLE

Co-expression of LPHN3 and NKX3.1 as a predictive biomarker for metastasis in breast cancer

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Abstract

Objective: To assess latrophilin-3 (LPHN3) and Homeobox protein (NKX- 3.1) protein expression in breast cancer, and the association with metastasis.

Method: The case-control study was conducted at the Dow University of Health Sciences, Karachi, and comprised breast carcinoma tissue sections diagnosed between July and November 2022. The tissues were divided into 2 equal groups, with group A having cases with metastasis and group B having controls without metastasis. expression of latrophilin-3 and NKX3.1 in tissue was assessed using immunohistochemistry in both the groups. Data was analysed using SPSS 21.

Results: Of the 80 women with mean age 50±1.20 years, 40(50%) were in each of the 2 groups. Overall, 31(38.8%) tissues were from the right-side breast, and 49(61.2%) were from the left-side breast. Latrophilin-3 was highly expressed in group A 35(87.5%) compared to group B 10(25%) ($p<0.001$). The expression of NKX3.1 was weak in 10(25%) group A cases and 4(10%) group B controls ($p=0.070$).

Conclusions: The expression of latrophilin-3 could play an important role in the prediction of metastasis in early-stage breast cancers. In contrast, NKX3.1 expression had no significant association with metastasis.

Key Words: Immunohistochemistry, LPHN3, NKX3.1, Breast cancer, Metastasis.

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Introduction

In recent times, breast cancer (BC) is known to be the leading cause of death among women. This specific malignancy is the major cause of morbidity in many developing countries, including Pakistan, and the main reason is advance stage of cancer at the time of diagnosis.[1]

Amongst the diseases disturbing human population worldwide, major burden of mortality is because of cancer and the female BC has transcended the lung cancer as the most diagnosed cancer globally (24.9%).[2] The major contributor to this burden is Asia (45.5%), and within Asia Pakistan's contribution is around 2.5%.[3] It is estimated that in every 9 women, one shall develop BC during her lifetime.[4]

Anatomically, breast tissue undergoes significant modifications throughout the life of a female because of numerous events, including puberty, pregnancy and lactation. Understanding of cancer progression and its metastasis is vitally related to these modifications of normal tissue that change the microenvironment and may lead to the origination of malignant cells. The classification of breast malignancy is done on the basis of histopathological features as well as on molecularity, and all these types differ with respect to survival rate and clinical expressions.[5,6] The metastasis of breast malignancy is defined as the final stage of progression as a result of number of steps, including circulation, invasion, angiogenesis and increase in the size of initial tumour.[7,8] Regional metastasis to axillary lymph nodes or distant organ metastasis occurs in invasive types of breast malignancies, and it is said to be significantly life-threatening. Hence, multidisciplinary effort is required for the prompt diagnosis and management of carcinoma, including investigations, surgeries and therapies.[9,10]

Most BC cases, especially young females, present and are diagnosed at a very late stage in Pakistan.[11,12] Information related to numerous factors, like the location of tumour, grade and size of tumour, subtype of tumour and the status of metastasis, is essentially important to completely evaluate BC so that the right management could be provided.[12,13] The microenvironment is one of the most significant factors that contribute to BC, but investigations that reveal the changes occurring in the microenvironment are not part of routine diagnostic tests.[14] Knowledge of changes occurring in the breast during the formation of malignant cells or during metastasis may help significantly in early or accurate

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detection of the disease or its aggressiveness.[15]

Several studies have stated that disturbed cell cycle can contribute to cancer development considerably.[16] The latrophilin-3 (LPHN3) gene comprises a number of cell adhesion molecules, like cadherin, immunoglobulin G (IgG), laminin A, thrombospondin type 1, galactose, lectin, Epidermal growth factor (EGF), besides transmembrane segments that are also included in signalling intracellularly at the time of cell-to-cell adhesion. The significance of this gene in specific diseases, like brain ischemia or attention deficit disorder, has been studied, but its role in cancer, specifically BC or its metastasis, is not well listed.[17]

Another gene, known as NKX3.1 and specific for prostatic tumour suppression, is located on chromosome 8p.[18] It is an androgen-regulated homeodomain gene, predominantly localised to the epithelium of prostate, and its contribution to the detection of prostatic origin among metastatic adenocarcinoma has been established.[19] The importance of NKX3.1 expression as an androgen-regulated tumour suppressor gene in BC is indirectly associated with the presence of oestrogen and androgen receptors. However, its contribution in BC metastasis is also a researchable domain.[20]

Recent research has investigated the roles of LPHN3 and NKX3.1 in relation to hormone receptors, such as progesterone receptor (PR) and oestrogen receptor (ER), even though NKX3.1 is known to block ER signalling, which may influence the behaviour of ER-positive breast tumours. It is yet unknown how LPHN3 precisely interacts with ER and PR in BC. Understanding the complexity of BC and creating successful treatment plans depend on how these indicators interact with hormone receptor status.[21]

The current study was planned to assess LPHN3 and NKX3.1 expression in BC, and the association with metastasis.

Materials and Methods

The case-control was conducted at the Dow University of Health Sciences (DUHS), Karachi, and comprised BC tissue sections diagnosed between July and November 2022. The tissues were divided into 2 equal groups, with group A having cases with metastasis and group B having controls without metastasis. The sample size of 65 tissue samples were calculated using PASS version 15, based on chi-square test with 95% confidence of interval and 80% power of test, effect size of 0.502 with degrees of freedom 10 computed using association between LPHN3 expression in all the types of breast tissues.[22] To increase the study validity, total 80 sample tissues were recruited.

After approval from the institutional ethics review committee, the specimens were obtained from the Histopathology section of the Dow Diagnostic Reference and Research Laboratory (DDRRL), Karachi. The samples included those sent from various collection points present throughout the province of Sindh. The samples were collected using non-probability purposive/simple random sampling technique.

Only biopsy-proven BC cases of all stages were included from mastectomy, lumpectomy or core biopsy procedures. Specimens related to patients treated with chemotherapy or radiotherapy prior to the sample collection or those who had BC recurrence were excluded. All the specimens were diagnosed and classified as per the World Health Organisation (WHO) classification.[23]

The specimens were subjected to immunohistochemistry (IHC) for LPHN3 and NKX3.1 detection. Primary antibody incubation for approximately 60 minutes in optimum dilution at room temperature was done (Envision+, Dako, Denmark). Two sections obtained from each of the patient were deparaffinized and then rehydrated. After blocking the endogenous peroxidase with 3% hydrogen peroxide (H2O2) for approximately 10 minutes, the sections were washed. Slides were cooled for 15-20 minutes. If retrieval was done, sections were incubated for 1 hour separately with two different antibodies. Then washing with Phosphate-buffered saline (PBS) was done 4 times. The detection of primary antibodies was done by the process of staining which included incubating with diaminobenzadine (DAB) and its enhancer for 5-15 minutes. Then the sections were washed in tap water. After that, counterstaining was done with Harris haematoxylin for 5 minutes, and finally dehydration was done with clearing so the sections were ready for visualisation.

A standard grading system based on glandular/tubular differentiation[3], nuclear pleomorphism[3] and mitotic rate[3] was used to score histological grades of BC as well-differentiated tumours grade I, moderately-differentiated tumours grade II and poorly-differentiated tumours grade III.[17, 24]

The IHC index of LPHN3 was categorised as >4 high) or <4 low on the basis of expression score ranging 1-9, which was calculated by multiplying the score of percentage and intensity of LPHN3 positively in the malignant cells. This was done on the basis of literature as 1-25% = 1, 26-50% = 2 and >50% = 3. In addition, the intensity was scored as 1 = low, 2 = moderate and 3 = high.[25]

Data was analysed using SPSS 21. Mean ± standard

deviation were calculated for quantitative variables, while frequencies and percentages were calculated for qualitative variables. Stratification was done and data was checked for normality, which was not normally distributed. As such, independent t-test was applied for mean comparison. Chi-square test was used to detect the effect modifiers on outcome. P≤0.05 was considered significant.

Results

Of the 80 women with mean age 50±1.20 years, 40(50%) were in each of the 2 groups. Overall, 31(38.8%) tissues were from the right-side breast, and 49(61.2%) were from the left-side breast. There were 66(82.5%) Ductal type BC cases, while 14(17.5%) were Luminal type. There were 48(60%) cases with grade II disease, followed by 31(38%) of grade III, and 1(1.2%) of grade I. IHC score was 5 in 1(1.2%) case, 6 in 23(28.8%) cases, 7 in 29(36.2%) cases and 8 in 27(33.8%) cases. In group A, 15(37.5%) cases were locally metastasised, while 25(62.5%) were with distant metastasis (Table 1).

Table-1: Patient characteristics

Patient characteristics	Frequency (%)
Mean Age (years)	50.93±1.20
Breast Cancer Site	
Right Side	31 (38.8)
Left Side	49 (61.2)
Breast cancer type	
Ductal	66 (82.5)
Luminal	14 (17.5)
Grade	
Grade I	1 (1.2)
Grade II	48 (60.0)
Grade III	31 (38.8)
Cancer Score (Bloom and Richardson)	
5	1 (1.2)
6	23 (2.8)
7	29 (36.2)
8	27 (33.8)
Metastatic level	
Locally spread	15 (18.8)
Distant Metastasis	25 (31.2)

SD: Standard deviation.

LPHN3 was highly expressed in group A 35(87.5%) compared to group B 10(25%) (p<0.001). The expression of NKX3.1 was weak in 10(25%) group A cases and 4(10%) group B controls (p=0.070).

Associations of different features of cancer with the expression of lphn3 and nkx3.1: To build up associations between the features of participants like age and characteristics of cancer like site, type, grade or level of metastasis, we applied Pearson chi-square. We found the positive association between the expression of LPHN3 and level of metastasis (locally spread/distant metastasis) with significant p-value with Representative microphotograph at 40 X of Breast cancer cases with metastasis. (A: H&E slide B: IHC Expression of LPHN3. C: IHC Expression of NKX3.1) (Table:2 & Figure: 1).

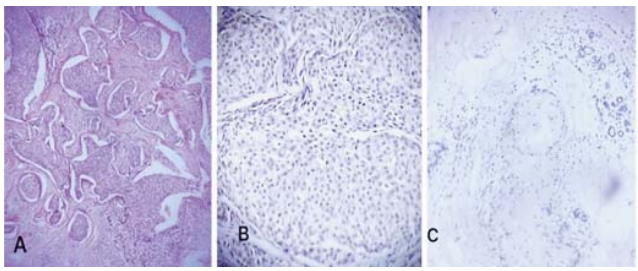

Figure: Micrographic presentation of latrophilin-3 (LPHN3) and NKX3.1 expressions Representative microphotograph at 40X of breast cancer cases with metastasis.
A: Haematoxylin and eosin (H&E) slide B: Immunohistochemistry (IHC) expression of LPHN3. C: IHC expression of NKX3.1.

Table-2: Association of different features of cancer with IHC expression.

Features		LPHN3 Expression		NKX3.1 Expression		P-value	
		Positive(%)	Negative(%)	Positive(%)	Negative(%)	LPHN3	NKX3.1
Side Of Cancer	Right	24(49.0)	25(51.0)	8	41	0.769	0.112
		21	10	6	25		
Type Of Cancer	Ductal	38(57.6)	28(42.4)	9(13.6)	57(86.4)	0.768	0.063
	Luminal	7(50)	7(50)	5(35.7)	9(64.3)		
Metastasis level	Locally Spread	13(86.7)	2(13.3)	4(26.7)	11(73.3)	<0.001	0.2
	Distant Metastasis	22(88.0)	3(12)	6(24.0)	19(76)		
Grade Of Cancer	1	1(100)	Nil	Nil	1(100)	0.388	0.596
	2	29(60.4)	15(48.4)	10(20.8)	38(79.2)		
	3	19(39.6)	16(5.16)	4(12.9)	27(87.1)		
Score (Bloom and Richardson)	5	1(100)	Nil	1(100)	Nil	0.787	0.206
	6	13(56.2)	10(43.5)	7(30.4)	16(69.6)		
	7	17(58.6)	12(41.4)	4(13.8)	25(86.2)		
	8	14(51.9)	13(48.1)	2(7.4)	25(92.6)		
Age of the patients	<40	27(61.4)	17(38.6)	8(18.2)	36(81.8)	0.368	1.00
	>40	18(50.0)	18(50.0)	6(16.)	30(83.3)		

LPHN3: Latrophilin-3, IHC: Immunohistochemistry.

In the current study, all NKX3.1 positive cases were also positive for ER and PR positive, while 2 LPHN3-positive cases showed ER and PR positivity.

Discussion

BC, the most prevalent and fatal carcinoma in premenopausal and

postmenopausal women, exhibits a wide range of age-related incidence[26] and significant ethnic diversity.[27] Numerous studies have indicated a steady increase in its incidence in Pakistan.[13] In a study in Islamabad, the average age of BC cases was 46.04+/-10.62 years.[28] Another study using data from 10,018 patients in Pakistan showed that the majority of instances were noted in women in their 40s.[29] Premenopausal and postmenopausal BC women residing in Karachi were included in the current study, and their ages ranged 25-75 years with a mean of 50.92 years.

In the current study, left-side case were more than the right-side Ones, while a study with 290 BC patients reported that majority of cases were on the right side and in the upper quadrant of the breast.[30] Also, the majority of cases in that study were in advanced stages (stages III and IV) when diagnosed.[30] According to a study in Pakistan, the majority of malignancies were of stage II at the time of diagnosis.[29] In the current study, the majority of BC diagnosis occurred in stages II and III. In addition, a higher proportion of patients had an advance cancer score, and the cancer was severely metastatic in almost one-third of the patients, indicating that the diagnosis had been made very late. This could be due to a variety of factors, including lack of knowledge or a lack of early-stage diagnostic options.[4,31]

Ductal BC was the most common BC type in the current study, which was in line with literature.[28] The importance of targetting the immune system with various approaches has been studied, introducing various immunotherapies that primarily target interaction between cancer cells and immune cells to maximise benefits related to BC treatment.[32]

The role of the immune system in order to prevent or recognise the progress of malignancy is well-established, specifically in BC. In view of the persistent need to find new antigens for BC immunotherapies, the significance of LPHN isoforms in preventing anti-tumour immunity was examined in a study.[33] Another study assessed its expression, especially in breast tissue, whether normal or malignant, and built up a link between it and the occurrence, maintenance and advancement of BC. This makes the protein an intriguing target that has to be looked at.[34]

Whether BC is metastatic or not, the current study noted a positive expression of LPHN3 in the BC tissue. Although LPHN3 was expressed in all BC cases, there was a notable variation in the degree of expression between metastatic and non-metastatic BC. As a result, its expression could act as a protein that may predict metastasis. By using reverse transcription polymerase chain reaction (RT-PCR) to detect axillary node metastases, a strong correlation between LPHN3 and matrix metallopeptidase 13 (MMP13) messenger ribonucleic acid (mRNA) expression was discovered, supporting the findings of the current investigation.[17] However, a study in Thailand found that compared to BC tissues with positive axillary lymph nodes (LNs), those with negative axillary LNs had higher levels of LPHN3 expression. In contrast, the current findings indicated that LPHN3 was much more expressed in BC cases with metastatic disease than in cases without metastatic disease ($p<0.001$).

As such, the findings regarding the expression of LPHN3 and its relationship with metastasis remain inconclusive, and more research in this area is required, either using larger sample sizes or various methodologies.

In the current study, the role of NKX3.1, another protein with a well-established and acknowledged involvement in the development of the male prostate gland, was investigated. It has been the most accurate prostatic origin detector in metastatic malignancies and is recognised to be the first particular marker associated with the prostate epithelium.[35] Studies have revealed that NKX3.1 may be used as a marker in the assessment of a variety of malignancies, including salivary duct carcinomas.[36] A study evaluated 349 non-prostatic tumours in total, but only one of these, invasive lobular carcinoma (ILC) of the breast,[27] tested positive for the NKX3.1 gene. Similar results were obtained in the current study.

The IHC expression of NKX3.1 protein was assessed in a study in which BC instances linked to ER, PR and human epidermal growth factor receptor 2 (HER2) were characterised. It was discovered that 27% of ILC and only 2% of invasive ductal carcinoma (IDC) had noticeable positive staining.[28] The current study showed that, despite their higher frequency than previously reported, lobular BC (35.7%) expressed NKX3.1 at a higher level than ductal BC (13.6%). Additionally, this characteristic was emphasised in a different study[37] that focussed on the distinctions between lobular and ductal BC. The study hypothesised that lobular BC was the only condition in which NKX3.1 was expressed.

In the current study, all NKX3.1-positive cases were also ER and PR positive, which was in concordance with an earlier study.[38] No study was found which could have highlighted the association of LPHN3 status with ER/PR positivity. The current study has limitations as it was conducted at a single centre and had a small sample size.

Conclusion

BC tissue expressed both LPHN3 and NKX3.1. The LPHN3 expression was strongly linked to metastasis, and could act as a biomarker for BC patients, allowing early detection and aggressive treatment. NKX3.1 was considerably less frequently expressed in BC tissue.

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AUTHORS' CONTRIBUTIONS:
RK: Principal investigator, design, concept, writing IRB for approval of study and project administration.
MA: Concept, design and supervision.
NK: Investigate the cases, supervision and implementation of experimental protocol.
MBK: Data collection, analysis, interpretation and drafting.
FFA: Critically reviewed, drafting, implementation of critical experimental protocol and publishing the research study.
SK: Statistical analysis, proper implementation of experimental work and drafting.