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Research Paper

Immunohistochemical Evaluation of ALK Expression in Breast Cancer

Medical Science

KEYWORDS : Breast cancer, ALK , Immunohistochemistry

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ABSTRACT

Extensive studies are conducted to identify factors involved in breast cancer genesis and to understand the genetic and cellular mechanisms which can lead us to new treatments. ALK was originally discovered as the underlying factor in the pathogenesis of anaplastic large cell lymphomas. ALK is believed to foster tumor genesis following activation by autocrine and/or paracrine growth loops involving ALK ligands. Expression of ALK has been shown in a variety of tumors including rhabdomyosarcoma, neuroblastoma and neuroectodermal tumor, glioblastoma, melanoma and inflammatory breast cancer. 61 cases of female breast cancer were evaluated immunohistochemically for the expression of ALK in the malignant epithelial cells and adjacent non-neoplastic tissue, and for correlation of expression with clinicopathological parameters as; age, tumor size, tumor type, tumor grade, tumor stage, lymph node status, dermal lympho-vascular embolization, degree of lymphocytic infiltration and ER, PR, Her-2 neu expression and molecular types. Eighteen cases out of 61 (29.5% of all cases) showed positive cytoplasmic expression for ALK in malignant epithelium. 8 positive cases showed concomitant cytoplasmic and nuclear immunoreactivity. ALK expression was observed in normal structures as breast epithelium, lymphocytes, nerves and fibroblasts. Expression in normal breast epithelium was localized to the nucleus without cytoplasmic localization. The current study found non-significant correlation between malignant epithelial ALK expression and various clinicopathological parameters, ER, PR, Her-2 neu expression and molecular types. However the correlation between ALK expression and histological type was close to significance (0.08), implying slight higher tendency for ALK expression among ductal carcinoma whether in pure or mixed forms. The issue of involvement of ALK in pathogenesis and progression of breast cancer needs thorough evaluation, especially by molecular techniques. Up till now no established link has been detected in breast cancer various types including inflammatory breast carcinoma. The availability of anti-ALK protein drugs however encourages further studies to enroll patients into clinical trials evaluating ALK targeted therapeutics.

Introduction

Worldwide, breast cancer is the most common invasive cancer in women [1]. In 2008, breast cancer caused 458,503 deaths worldwide; 13.7% of cancer deaths in women and 6.0% of all cancer deaths for men and women together [2]. Today, extensive studies are ongoing to identify factors involved in breast cancer genesis and to understand the genetic and cellular mechanisms which can lead us to new treatments [3].

The ALK TK receptor gene is located at 2p23.2 [4] and belongs to the insulin receptor superfamily. The structure of this single chain transmembrane receptor consists of an extracellular domain containing an N-terminal signal peptide sequence, together with the binding sites for the activating ligands of ALK; pleiotrophin, and midkine [5]. ALK was originally discovered as the underlying factor in the pathogenesis of anaplastic large cell lymphomas (ALCL) [6]. ALK normally has a restricted distribution in mammalian cells, being found at significant levels only in the nervous system during embryonic development. The protein decreases in its expression during gestation and in adult mammals ALK expression is limited to rare neural cells and scattered pericytes and endothelial cells after birth [7].

ALK is believed to foster tumor genesis following activation by autocrine and/or paracrine growth loops involving the reported ALK ligands, pleiotrophin and midkine [8]. Expression of ALK has been shown in a variety of tumors including rhabdomyosarcoma [9], neuroblastoma and neuroectodermal tumor [10], glioblastoma [11] and melanoma [12]. In addition, ALK gene amplification is a common feature of inflammatory breast cancer [13].

Point mutations of ALK TK receptor gene have been found in 6 to 8% of primary neuroblastoma. Germ-line mutations have been identified in families with more than 1 sibling with neuroblastoma. Somatic mutations with wild-type ALK in matched constitutional DNAs have also been described in nonfamilial neuroblastoma cases. These mutations are located mainly in the TK domain; the most frequent being the gain-of-function mutations F1174L and R1275Q. These mutations are associated with increased expression, phosphorylation, and kinase activity of the ALK protein. Further, they have been shown to have Ba/F3 cell-transforming capacity. In some cases, these mutations coexist with an increased copy number of the ALK gene [14].

Therapeutic approaches consisting of gene therapy and immunotherapy targeting this molecule hold promise [15], among them Crizotinib can be noted [16]. Although initially designed against the hepatocyte growth factor receptor (c-Met), Crizotinib (PF-2341066) is a potent, ATP-competitive, small molecule inhibitor of ALK phosphorylation and its oncogenic variants, available in oral formulation [17].

Our Study aims to analyze the expression of ALK in tissues derived from human breast cancer using immunohistochemistry, compare expression patterns with adjacent non-neoplastic tissue and correlate expression with prognostic clinicopathological factors of all cases to raise the possibility that constitutively activated ALK oncoprotein may contribute to the pathogenesis and progression of breast cancers and thus can be a target of immunotherapy and gene therapy.

Materials And Methods

Study design

Retrospective cross-sectional study where tumor tissue samples were obtained from paraffin blocks of 61 breast cancer female patients, who underwent radical mastectomy or wide excision and axillary clearance, between January and December 2013 at a private laboratory. These tumor tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Clinical data of the patients were collected from the files (age), as well as histopathological data (tumor size, tumor type, tumor grade, tumor stage, lymph node status, dermal lympho-vascular embolization, degree of lymphocytic infiltration).

Histopathological study

Formalin-fixed paraffin embedded sections (4 µm thick) were mounted on glass slides and subjected to H&E staining, and

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then slides were examined pathologically to revise histopathological type, tumor grade, presence of lympho-vascular emboli and extent of lymphocytic infiltration. The already available ER, PR and Her2 immunohistochemical staining status for each case were retrieved from patient's files (initially done as a routine prognostic and theraputic step).

The state of estrogen and progesterone immunoreactivity according to Quick score system was recorded from patient's files. Patients with scoring 2 or less are regarded as ER and PR negative and those with scoring above two are regarded as ER and PR positive. Immunohistochemical staining for HER2 was recorded as well. A score of 2+ or 3+ was regarded as 'positive' and a score of 0 or 1+ as 'negative'.

Immunohistochemical staining

The immunohistochemistry study was done by using DAKO Monoclonal Mouse Anti-Human CD246 (ALK) Ab (DAKO, Denmark). Tissue sections from paraffin-embedded blocks were placed on charged slides, deparaffinized in xylene then rehydrated in descending alcohol concentrations. Endogenous peroxidase was blocked by incubating the sections with 3% hydrogen peroxide and the tissues were permeabilized by incubating the samples in Tris-buffered saline (TBS, pH 7.6) with 1% Triton X-100. Slides were placed in EDTA unmasking solution (pH adjusted to 8.0) into the pressure cooker. 100–300 µl primary antibody diluted according to the product datasheets (dilution ratio: 2/100, pH: 6) and added to each slides, immersed for 40 minutes. 1–2 drops of EnVision™+ were added to each section at room temperature. 100–400 µl DAB reagent (staining solution) was added to each section for 10-15 minutes. Upon completion of development, slides were counterstained in hematoxylin. Finally they were dehydrated in alcohol, cleared in xylene and mounted. The slides between successive steps were rinsed in wash buffer.

Semi-quantitative subjective assessment of two independent observers was conducted in this study. The staining of the tissue sections was evaluated by two investigators. ALK expression pattern, intensity and extend were assessed. Positivity of expression was considered with moderate or intense staining in more than 10% of tumor population; equivalent to score (2+) or (3+) in some other series. Absent staining, staining in less than 10% or faint staining in more than10% of tumor population, were considered as negative tumor expression; equivalent to score (0) or (1+) in some other series. A section of immunostained anaplastic large cell lymphoma was utilized as external positive control and nerve bundles positive immunostaining as positive internal control.

Statistical analyzes

IBM SPSS software version 20 was used for all statistical analyses. Associations between ALK status and other clinicopathological parameters were assessed. For quantitative variables mean and standard deviation were used, and for qualitative ones prevalence and ratio were used. Means were compared with paired student t-test. Pearson Chi square test and Fisher exact's test were used to detect significance of correlations, 2-sided significance for each correlation was considered. Generalized Linear Models; GLM (UNIANOVA) was used to compare two groups (test of between subject effects). All P-values of <0.05 resulting from two-sided tests were considered significant.

Results

In this study the total cases were 61 female breast cancer patients, whose age range was between (29-70) years with mean age of 52.1 years ± 10.8. Invasive duct carcinoma constituted 45 (73.8%) of all the studied cases, while invasive lobular and mixed carcinoma (lobular and duct) constituted 8 cases each (13.1%). Tumor sizes ranged from 1 to 11 cm with a mean of 4.25 cm ± 2.5. Tumor grade II constituted the majority of all the studied cases, 52 (85.2%) while grade III were 9 cases (14.8%). As regards tumor stage, 8 cases were T1 (13.1%), 32 cases were T2 (52.5%), 11 cases were T3 (18%) and 10 cases were T4 (16.4%). Forty five cases had lymph node metastasis (73.8%), N1 were 15 cases (24.6%), N2 were 13 cases (21.3%) and N3 were 17 cases (27.9%). Dermal vascular emboli were seen in only 6 cases (9.8%), but not seen in the majority of cases; 55 (90.2%). The intensity of lymphocytic infiltration was either mild in 44 cases (72.1%), moderate in 13 cases (21.3%) or marked in 4 cases (6.6 %). ER positive expression was detected in 43 (70.5%) of total cases and was absent in 18 cases (29.5%). PR positive expression was detected in 35 cases (57.4%) and was absent in 26 cases (42.6%). HER2 positive expression was detected in 14 (23%) of total cases and was absent in 47 cases (77%). Based on hormone receptor immunoreactivity, 43 (70.4%) cases were of luminal type, 11 (18%) were triple negative and 7 (11.6%) were Her-2 enriched.

Eighteen cases out of 61 (29.5% of total cases) showed positive cytoplasmic expression for ALK in malignant epithelium, 14 of positive cases were invasive duct carcinoma NOS; representing 31.1% of total NOS cases, 2 were invasive lobular and 2 were mixed ductal and lobular; representing 25% of total cases in either group types (fig. 1). 8 positive cases showed concomitant cytoplasmic and nuclear immunoreactivity. ALK expression was observed in all cases as regarding normal structures in breast epithelium (fig. 2), lymphocytes, nerves and fibroblasts. Expression in normal breast epithelium was localized to the nucleus without cytoplasmic localization (fig. 2). The distribution of the various clinicopathological parameters and hormonal receptor status among each of ALK positive and negative groups is illustrated in both table 1 and table 2.

The correlation between malignant epithelial ALK expression and various clinicopathological parameters; age, tumor size, tumor type, tumor grade, tumor stage, lymph node status, dermal lympho-vascular embolization, degree of lymphocytic infiltration; as illustrated by different statistical tests were all non-significant (table. 1). However the p-value of the correlation between ALK expression and histological type was close to significance (0.083), implying slight higher tendency for ALK expression among ductal carcinoma whether in pure or mixed forms. The correlations between ALK expression and ER, PR, Her-2 neu expression and molecular types were all as well non-significant (table. 2).

Table 1: Correlation between ALK expression and other clinicopathological parameters.

	ALK expression			
	Negative=43 (100%)	Positive=18 (100%)		
Factors	N (%)	N (%)	P value	Significance
Age (yrs.)				
Mean ±SD	53±10.4	50.2±11.97	0.481*	NS
Tumor Size(cm)				
Mean ±SD	4.33 ± 2.6 cm	4.07 ± 2.3 cm	0.87*	NS
Histological type				
Duct Carcinoma	31 (72%)	14 (77.8%)	0.083 °	NS
Lobular carcinoma	6 (14%)	2 (11.1%)		
Mixed type	6 (14%)	2(11.1%)		
Grade				
II	37 (86%)	15(83.3%)	0.500 α	NS
III	6 (14%)	3(17.6%)		
Tumor Stage				
T1	6 (14%)	2 (11.1%)	0.238 °	NS

T2	23 (53.5%)	9 (50%)		
T3	7 (16.3%)	4 (22.2%)		
T4	7 (16.3%)	3 (16.7%)		
Nodal Metastasis				
-Ve	14 (32.6%)	2(11.1%)	0.500 α	NS
+Ve	29 (67.4%)	16(88.9%)		
N Stage				
N0	14 (32.6%)	2 (11.1%)	0.213 *	NS
N1	11 (25.6%)	4 (22.2%)		
N2	6 (14%)	7 (38.9%)		
N3	12 (27.9%)	5 (27.8%)		
Dermal Emboli				
-Ve	38 (88.4%)	17 (94.4%)	0.500 α	NS
+Ve	5 (11.6%)	1 (5.6%)		
Lymphocytic Infiltrate				
Mild	0 (0%)	4 (22.2%)	0.199 *	NS
Moderate	35 (81.4%)	9 (50%)		
Marked	8 (18.6%)	5 (27.8%)		

***Paired student t-test, α Fisher's exact test, * Pearson Chi square test**

Table 1: Correlation between ALK expression and hormonal receptor statuses.

	ALK expression			
	Negative=43 (100%)	Positive=18 (100%)		
Factors	N (%)	N (%)	P value	Significance
Estrogen receptors				
-Ve	14 (32.6%)	4 (22.2%)	0.500 α	NS
+Ve	29 (67.4%)	14 (77.8%)		
Progesterone receptors				
-Ve	19 (44.2%)	7 (38.9%)	0.500 α	NS
+Ve	24 (55.8%)	11 (61.1%)		
Her2/neu				
-Ve	10 (23.2%)	4 (22.2%)	0.500 α	NS
+Ve	33 (76.8%)	14 (77.8%)		
Molecular types				
Luminal	29 (67.4%)	14 (77.8%)	0.214 β	NS
Triple negative	9 (21.1%)	2 (11.1%)	0.361 β	NS
Her-2 enriched	5 (11.5%)	2 (11.1%)	0.258 β	NS

α Fisher's exact test, β UNINOVA (GLM)

Fjgure (1): Invasive duct carcinoma (NOS) showed positive expression of ALK.

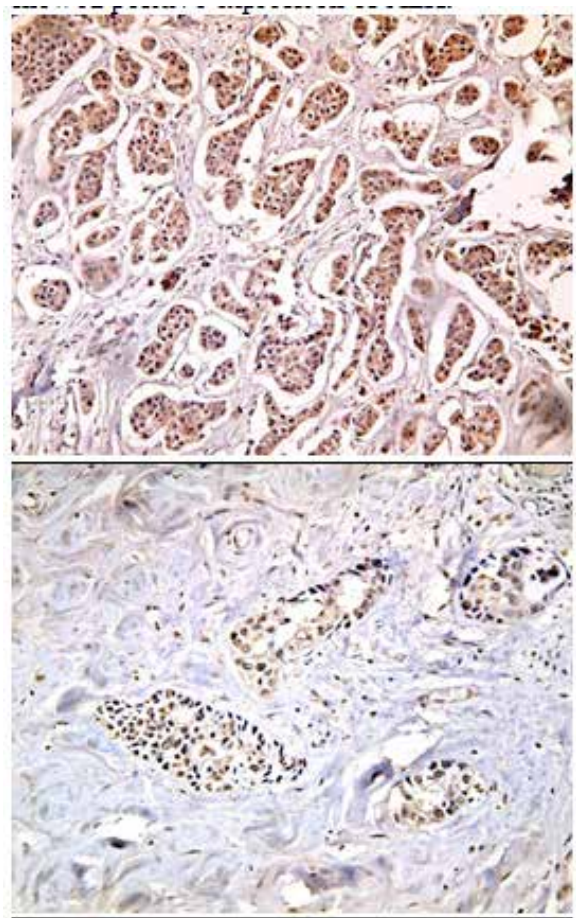
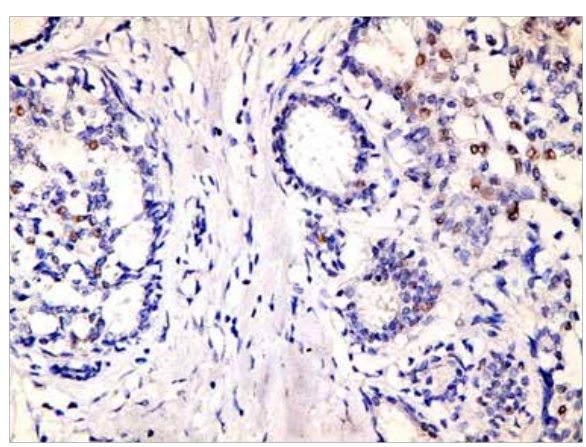


Figure (2): Normal breast lobule with positive nuclear ALK expression in the acinar epithelium.



Discussion

Breast cancers progress through genetic and epigenetic mutations that deregulate oncogenic pathways that initiate a more aggressive breast cancer phenotype [18]. The identification of these mutations is of major importance, since, once identified, it is likely their identification will lead to new targets for therapeutic development and potentially target directed therapy [3].

The current study demonstrated positive expression of ALK oncoprotein in 18 cases out of 61 (29.5%). 14 of positive cases were

invasive duct carcinoma NOS; representing 31.1% of total NOS cases, 2 were invasive lobular and 2 were mixed ductal and lobular; representing 25% of total cases in either group types. This extend of positive expression is much lower than that reached by Perez-Pinera et al. [3] who demonstrated that 75% of the infiltrating ductal carcinomas, 50% of the infiltrating lobular carcinomas showed positive ALK expression. Also Mehrjardi and Vaghefi [8] found that ALK was expressed in 47% of breast cancer sections analyzed where all 8 invasive lobular carcinoma were ALK-negative and about 50% of invasive ductal carcinomas (NOS type) and medullary carcinomas were ALK-positive. Such decline in our study may be explained by variability of molecular and genetic pathogenesis at different geographic locations or by differences in section immunohistochemical processing, different fixation method and tissue processing, different kinds of antibody used for detection of ALK protein or by difference scoring system applied where both included samples with weak staining (+1) which we excluded from positive cases in our study.

Our study revealed cytoplasmic staining in all positive cases with 8 cases showed concomitant cytoplasmic and nuclear immunoreactivity. ALK expression was observed in all cases as-regarding normal structures in breast epithelium, lymphocytes, nerves and fibroblasts. This was in concordance with Perez-Pinera et al. [3] who found that ALK was expressed in the breast cancer cells localized to the cytoplasm and nuclear expression was found in lobular carcinoma and some cases of medullary type, and in all cases analyzed ALK also was expressed in the tumor associated fibroblasts within the foci of breast cancer cells, nerve fibers, in normal breast epithelium and in the smooth muscle cells of the blood vessels. Also Mehrjardi and Vaghefi [8] found ALK was localized in cytosol and nuclei as well as cell membrane in breast cancers.

Normal breast epithelium in our study showed nuclear ALK expression only. This finding implies that cytoplasmic localization in malignant epithelium might be related to aberrant ALK expression by mutation or amplification. This is in contrast to both Perez-Pinera et al. [3] and Mehrjardi and Vaghefi [8] who described the distribution of ALK in normal breast epithelial cells to be localized to the cytoplasm, with higher expression in the apical surface in direct relationship to the ductal lumen. This point needs further evaluation with correlation to aberrant molecular alternations, where different aberrant genetic and molecular pathways associated with overexpression of ALK might be associated with different patterns of cytoplasmic, nuclear and cell membrane immunoreactivity.

The current study found non-significant correlation between malignant epithelial ALK expression and various clinicopathological parameters; age, tumor size, tumor type, tumor grade, tumor stage, lymph node status, dermal lympho-vascular embolization, degree of lymphocytic infiltration, ER, PR, Her-2 neu expression and molecular types. This was in concordance with results of Mehrjardi and Vaghefi [8] who found no relationship between ALK expression and patient's age, tumor type and grade, presence of necrosis, vascular invasion, skin involvement, lymph node metastasis and status of hormone receptors.

However the p-value of the correlation between ALK expression and histological type in our study was close to significance (p=0.083), implying slight higher tendency for ALK expression among ductal carcinoma whether in pure or mixed forms.This point needs further evaluation by molecular studies for ALK mutations or amplifications, as this might be involved in the genetic alternation involved in the emergence of ductal type breast carcinomas.

Conclusion

In conclusion, the issue of involvement of ALK in pathogenesis and progression of breast cancer needs thorough evaluation, especially by molecular techniques. Up till now no established link has been detected in breast cancer various types including inflammatory breast carcinoma. The availability of anti-ALK protein drugs however encourages further studies to enroll patients into clinical trials evaluating ALK targeted therapeutics.

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