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Original Article

Expression of Anaplastic Lymphoma Kinase Protein in Human Breast Cancer

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ABSTRACT

Background & Objectives: **Anaplastic lymphoma Kinase (ALK) is a tyrosine kinase receptor involved in the genesis of several human cancers. We aimed to examine ALK expression in different types of breast cancers and its relationship with tumor grade, tumor size, presence of necrosis, vascular invasion, skin involvement and lymph node metastasis.**

Material & Methods: **We examined a number of human breast cancers to see if ALK is expressed in this tumor and studied its relation with type of carcinoma and its grade, tumor size, presence of necrosis, vascular invasion, skin involvement, lymph node metastasis and patient's age. All statistical analyses were performed using SPSS version 15.0.**

Result: **Totally, 100 patients were enrolled with mean age of 50.2 ± 12.5 yr. The histological phenotypes of the breast cancers studied included invasive ductal carcinoma, invasive lobular carcinoma and medullary carcinoma. ALK expression was evaluated by immunohistochemistry which was positive in 47 cases (47%). No statistically significant relationship was found between the above mentioned parameters except for tumor size and ALK expression.**

Conclusion: **Anti-ALK protein drugs can be considered as a new therapeutic approach for breast cancer.**

Keywords: **Breast Cancer, Anaplastic Lymphoma, Tyrosine Kinase**

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Introduction

Breast cancer is the most common cancer (22% of all new cases) as well as the leading cause of cancer deaths (14% of all cancer deaths) in women worldwide. There have been sustained increases in the incidence of this cancer in developing countries in recent years (1). The incidence and the prevalence of breast cancer in Iranian women are 22 per 100,000 and 120 per 100,000 respectively, being most prevalent among 40-49 yr (2).

Today, extensive studies are ongoing to identify factors involved in breast cancerogenesis and to understand the genetic and cellular mechanisms which can lead us to new treatments (3). Among them anaplastic lymphoma kinase (ALK) gene could be cited. It is a tyrosine kinase receptor that is a member of the insulin receptor superfamily (3). It was originally discovered as the underlying factor in the pathogenesis of anaplastic large cell lymphomas (ALCL) (4-6). NPM–ALK results from the [2,5] (p23;q35) chromosomal translocation; however, other translocations can also lead to constitutive activation of ALK. ALK is a 1620 amino acid protein located along the cell membrane (7). ALK is believed to foster tumorigenesis following activation by autocrine and/or paracrine growth loops involving the reported ALK ligands, pleiotrophin (PTN) and midkine (MK).

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 (8). Expression of ALK has been shown in a variety of tumors including rhabdomyosarcoma (9), neuroblastoma and neuroectodermal tumor (10, 11), glioblastoma (12) and melanoma (13,14). In addition, ALK gene amplification is a common feature of inflammatory breast cancer (15).

Studies reveal that the presence of anti-ALK antibodies may be relevant to the relatively good prognosis in these patients (16). Therapeutic approaches consisting of gene therapy and immunotherapy targeting this molecule hold promise (17-19). Among them Crizotinib can be noted (20, 21).

In a study on 600 types of human cancer cells carrying ALK gene mutation, Crizotinib reduced cell proliferation (22). Two ALCL patients positive for the NPM-ALK fusion treated with single agent crizotinib exhibited complete responses (23). The use of this drug has also been effective in the treatment of advanced lung cancer (24). .

Since little research has been performed on the ALK expression in breast cancer, in this study we examined ALK expression in different types of breast cancers and its relationship with tumor grade, tumor size, presence of necrosis, vascular invasion, skin involvement and lymph node metastasis.

Materials and Methods

Initially, a group of 100 cases of primary breast cancer were included as a cross sectional study between March 2009 and February 2011 sent to the Pathology Laboratory in Firouzgar Hospital, Tehran. Eligible cases in this study consisted of radical mastectomy specimens, modified mastectomy and lumpectomy specimens.

Needle or incisional biopsy specimens were not enrolled in the study due to the lack of needed data. Sampling consisted of available specimen. Patients' identities were not registered and only the pathology codes were used.

Considering the first type of error, precision of

study and relative incidence of ALK expression equivalent to 5%, 0.1% and 70% respectively, sample volume was calculated to be 80 but for increasing the precision we took up to 100 cases.

We recorded patient's age, tumor type and grade, tumor size, status of necrosis, vascular invasion, skin and lymph node involvement and chose one appropriate paraffin-embedded tumoral tissue block for performing immunohistochemistry.

The immunohistochemistry study was done in Milad Hospital Pathobiology Laboratory by using DAKO Monoclonal Mouse Anti-Human CD246 (ALK) Ab (DAKO, Denmark) with the following instructions :

Tissue sections from paraffin-embedded blocks were collected on clean poly L-lysine coated glass slides, deparaffinized in the oven for 1-2 hours at 60 °C and then immersed in three washes of xylene for 10 minutes each, in two washes of 100% ethanol for 5 minutes each and in two washes of 95% ethanol for 5 minutes each. Then they were immersed twice in dH2O for 5 minutes each, and were immersed in hydrogen peroxide 1/10 with methanol for 15 minutes. The slides were rinsed twice for 5 minutes, and washed in wash buffer (TBS solution, including 160gr NaCl, 12gr Tris and 100cc distilled water, pH adjusted to 7.6 with concentrated HCl) for 5 minutes. Slides were placed in EDTA unmasking solution (0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2 \cdot 2H_2O$) to 1 L dH2O. pH adjusted to 8.0) into the pressure cooker, set for the Biocare Medical Decloaking Chamber follow (SP1 125°C 30 seconds and SP2 90°C 10 seconds) .

The slides were then let to cool on the bench for 10 minutes. Slides were rinsed with dH2O. 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. Antibody solution removed by placing sections in wash buffer three times for 5 minutes each. 1–2 drops of EnVision™+ added to each section and Incubate 30 minutes at room temperature. The slides placed in wash buffer three times for 5 minutes each. 100–400 µl DAB reagent (staining solution) was added to each section for 10-15 minutes. Upon completion of development, slides were immersed in dH2O, counterstained in hematoxylin for 15 seconds and washed in dH2O twice for 5 minutes each. Finally they were dehydrated in alcohol, cleared in xylene and mounted.

The levels of expression of ALK were scored according to the intensity of immunoreactivity observed in at least 10% of tumoral cells by light microscopy using a scale of 1 (low) to 3 (high). The staining was repeated in the cases we were unable to interpret as nonspecific staining in the whole section.

For quantitative variables, mean, median, standard deviation and for qualitative ones prevalence and ratio were used. Means compared with t-test and ratios with chi square test. P-values less than 0.05 were considered to be statistically significant. All statistical analyses were performed using SPSS version 15.0.

Results

Expression of ALK protein was tested in 100 samples of human breast using immunohistochemistry and its relationship with tumor grade, tumor size, presence of necrosis, vascular invasion, skin involvement and lymph node metastasis has been studied.

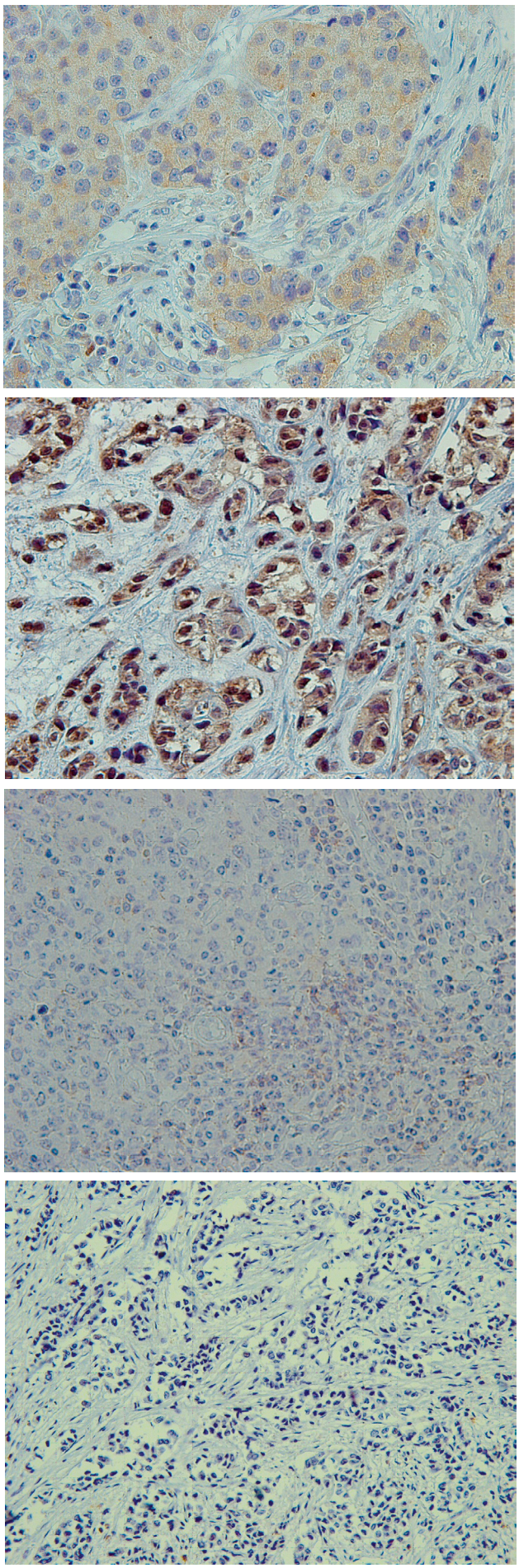
The mean age of the patients was 50.2 ± 12.5 years with the range of 27 to 87 years. The mean tumor size was 3.9 ± 2.4 cm with the range of 0.3 to 13 cm. Different tumors typed and their grade, status of necrosis, vascular invasion, skin and lymph node involvement as well as status of hormone receptors is shown in Table 1.

Table. 1- Characteristics of the 100 studied cases of breast carcinoma, including tumor type, tumor grade, necrosis, skin involvement, vascular invasion, lymph node metastasis, hormone receptors and ALK status.

Variable	Result
Tumor type (n=100)	
Invasive Ductal Carcinoma	88
Invasive Lobular Carcinoma	8
Medullary Carcinoma	4
Tumor grade (n=92)*	
I	7(7.6)
II	29(31.5)
III	56(60.9)
Necrosis (n=98)□	24(24.5)
Skin involvement (n=83)□	11(13.3)
Vascular invasion (n=100)	44(44)
Lymph node metastasis (n=96)□	68(70.8)
ER receptor positive (n=78) □	64(82.1)
PgR receptor positive (n=78) □	56(71.8)
Her-2/neu (n=78) □	
Negative	50(64.1)
+1	1(1.3)
+2	15(19.2)
+3	12(15.4)
ALK (n=100)	
Negative	53
+1	22
+2	12
+3	13

*For lobular carcinoma no grading was considered
□ Unreliable data were excluded
□ Done in another center and were not available

ALK was expressed in 47% of breast cancer sections analyzed (Fig. 1). It was is also expressed in samples of normal tissues (Fig. 1E). The pattern and intensity of expression of ALK was variable in different cells. ALK was localized in cytosol and nuclei as well as cell membrane in breast cancers. The results demonstrated that 13% of the breast carcinomas highly expressed ALK protein, 12% showed moderate reactivity, 22% mild and 53% revealed no staining.



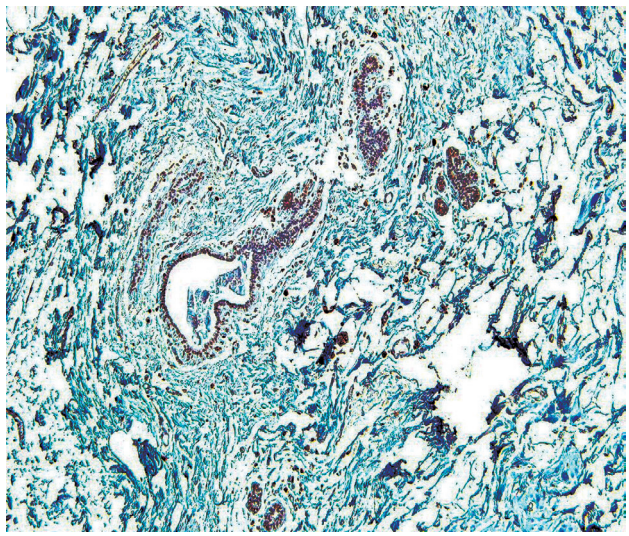


Fig. 1 - Different patterns of ALK expression in human breast cancers: A, Moderate Cytoplasmic and Weak Membranous expression pattern of ALK in an infiltrating ductal carcinoma(×400); B, Moderate Cytoplasmic and Strong Nuclear expression pattern of ALK in an infiltrating ductal carcinoma (×400); C, Negative in Medullary Carcinoma (×100); D, Negative in Invasive Lobular Carcinoma (×100); E, Normal Epithelium (×40)

Other findings of this study revealed that the mean age of the patients with ALK positive breast cancers was 48.6 ± 11.3 yr and the mean age of patients with ALK negative breast cancer was 50.3 ± 14.8 showing no significant difference(P=0.358).

Tumor size in ALK positive sample was 3.1 ± 1.8 cm and in ALK negative ones was 4.3 ± 2.4 cm with significant difference (P=0.043).

The relationship between intensity of ALK expression with tumor grade and hormone receptor status has been studied but the P value failed to reach a significant level (Table 2).

Table. 2- Relationship between intensity of ALK expression with tumor grade and hormone receptor status in the 100 studied cases of breast carcinoma

Variable	ALK				*P* Value
	Negative 53(%)	+1 22(%)	+2 12(%)	+3 13(%)	
Tumor Grade (n=92)					
I	5(62.5)	0	1(12.5)	2(25)	0.233
II	14(46.7)	7(23.3)	2(6.7)	7(23.3)	
III	31(55.4)	12(21.4)	9(16.1)	4(7.1)	
ER receptor (n=78)					
Negative	9(64.3)	4(28.6)	0	1(7.1)	0.328
Positive	36(56.3)	10(15.6)	6(9.4)	12(18.8)	
PgR receptor (n=78)					
Negative	12(54.5)	7(31.8)	1(4.5)	2(9.1)	0.188
Positive	33(58.9)	7(12.5)	5(8.9)	11(19.6)	

All 8 invasive lobular carcinomas were ALK-negative in our study. About 50% of invasive ductal carcinomas (NOS type) and medullary carcinomas were ALK-positive (Table 3).

Table 3. The relationship between other tumor characteristics, including tumor type, tumor grade, necrosis, skin involvement, vascular invasion, lymph node metastasis, Her-2/neu status and ALK expression

Variable	ALK		*P* Value
	Negative 53(%)	Positive 47(%)	
Tumor type (n=100)			
Invasive Ductal Carcinoma	51.1))45	43(48.9)	0.116
Invasive Lobular Carcinoma	4(100)	0	
Medullary Carcinoma	4(50)	4(50)	
Necrosis (n=98)			
negative	37(50)	37(50)	0.244
positive	15(62.5)	9(37.5)	
Skin involvement (n=83)			
negative	40(55.6)	32(44.4)	0.303
positive	7(63.6)	36.4))4	
Vascular invasion (n=100)			
negative	31(55.4)	25(44.6)	0.728
positive	22(50)	22(50)	
negative Lymph node metastasis (n=96)	60.7))17	27(44.2)11 (39.3)	0.518
positive	34(55.7)		
Her-2/neu (n=78)			
negative	30(60)	20(40)	
.+1	1(100)	0	0.409
.+2	8(53.3)	7(46.7)	
.+3	6(50)	6(50)	

Discussion

The results of our study reveal that ALK was expressed in 47% of breast cancer cases. The pattern and intensity of expression of ALK was variable in different tumors. Thirteen percent of the breast carcinoma cases highly expressed ALK protein. All 8 invasive lobular carcinoma were ALK-negative and about 50% of invasive ductal carcinomas (NOS type) and medullary carcinomas were ALK-positive. We 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.

The rate of ALK expression in breast cancers in our study differs somehow from the that reported by Perez-Pinera *et al.* (3) in which ALK was expressed in 75% of invasive ductal carcinomas and 50% of invasive lobular carcinomas. Similarly in their study ALK expression was different in pattern and intensity in different types of breast carcinoma. ALK was also expressed in samples of normal tissue in a pattern similar to our study, distributed to the cytoplasm with higher expression in the apical surface in direct relationship to the ductal lumen (Fig. 1E).These differences can be explained by different fixation method and tissue processing, different techniques in immunohistochemical staining including different kinds of antibody used for detection of ALK protein (25-28). Table 4 points out some of the differences in immunohistochemical staining method. Moreover it can be attributed to different genetic mutations that lead to breast carcinoma (22, 24). This discrepancy points to the need for more research.

Table 4. Comparison between our immunohistochemical staining method with Perez-Pinera *et al.* study in 2007(3)

	Perez-Pinera *et al.*	**Milad Hospital Pathobiology Laboratory**
Type of Method	LSAB	EnVision
Removal of Endogenous Peroxidase	H2O2 3% for 5 min	H2O2 1/10
Type of Antibody	Zymed, currently Invitrogen, Carlsbad, CA	DAKO Monoclonal Mouse Anti-Human CD246
Antibody Dilution Ratio	1/100	2/100
PH of Reaction	7.2	6
Antibody Incubation Time	overnight	40 min

In more recent studies, evaluation of ALK status in different tumor types like neuroblastoma (29) is essentially based on mutation analysis of ALK gene by molecular studies which can be another source of different results as expression of ALK gene related m-RNA was found in 73% of breast cancers in one study (4).

Paik *et al.* studied the correlation between screening of anaplastic lymphoma kinase rearrangement by immunohistochemistry in non-small cell lung cancer and fluorescence in situ hybridization. Immunoreactivity was scored as 0, 1, 2, or 3, and the results were compared with the FISH results. They find out that the sensitivity and specificity of IHC were 100% and 95.8%, respectively. Their data supported an IHC scoring algorithm in which ALK IHC scores of 0, 1, or 3 were highly compatible with FISH results, and

IHC scores of 2 were variable. They concluded that IHC assay using the 5A4 antibody reliably detected non-small cell lung cancer with ALK re-arrangement and might be useful as a screening method to identify these tumors (30).

However, given that ALK expression in breast carcinoma is a new subject and studies in this field have been done more on the presence of ALK mutation, not the expression of ALK protein, further studies should be directed to determine whether the presence of ALK gene mutations in breast cancer is associated with its expression. Similarly, it should be determined if anti-ALK protein drugs like crizotinib can also be effective in breast cancer. Finally, if preliminary clinical trials show that anti-ALK drugs are effective in breast carcinoma, then it is mandatory to verify the best sensitive yet cost-effective method to evaluate ALK status of individual breast tumors and to find out which staining pattern / intensity is an indicator of good response to anti-ALK drugs.

Conclusion

Anti-ALK protein drugs can be considered as a new therapeutic approach for breast cancers.

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