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Immunohistochemical Expression of ROS1 in invasive ductal carcinoma of breast in association with hormonal receptor status and Her2Neu expression

Muhammad Umair, Ahmed Ahson Khan, Nighat Jamal, Akhter Ali Bajwa, Tabish Hassan, Muhammad Umair Khan

ABSTRACT:

Objective: To determine the frequency of immunohistochemical expression of ROS1 in invasive ductal carcinoma of the breast in relation to hormonal receptor status and HER2 expression.

Study Design and Setting: Descriptive cross-sectional study. Department of Histopathology, Armed Forces Institute of Pathology, Rawalpindi from May 2022 to Dec 2022.

Methodology: This study was conducted on a sample size comprising 137 patients diagnosed with invasive breast carcinoma (ductal carcinoma) on histopathological biopsy specimen. Immunohistochemistry was performed using ROS1, estrogen receptor, progesterone receptor and HER2 antibodies on patients' tissue samples. Results were interpreted by two independent histopathologists. Finally data was analyzed using SPSS version 25.

Results: The mean age of sample population was 50.85 ± 12.17 years. 131 patients were women and 6 were men. ROS1 was positive in 54 cases. ROS1 shows weak staining in 41 cases and moderate to strong staining in 13 cases. ER and PR showed no significant statistical correlation with ROS1 expression. HER2 was positive in 37 cases, equivocal in 11 cases and negative in 89 cases. A significant statistical correlation was seen between ROS1 and HER2 as 23 of HER2 positive cases showed ROS1 expression (p=<0.001).

Conclusion: Significant number of ROS1 expressing cases in invasive breast carcinoma can be more revealing in the understanding of pathogenesis of breast carcinoma. In addition, it can also lead to use of certain recent tyrosine kinase inhibitors for treatment of this most common carcinoma in females.

Keywords: Invasive breast carcinoma, ROS1, Estrogen receptor, Progesterone receptor, HER2.

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INTRODUCTION:

In 2022, 2.3 million women were diagnosed with breast carcinoma and resulted in 670,000 deaths globally.[1] It is the most common type of cancer in women.[1] Pakistan is at the top of Asian countries with highest incidence of breast cancer where every one in nine women have a lifetime risk of being diagnosed with breast cancer.[2] Invasive breast carcinoma NST (ductal carcinoma) accounts for the majority of breast cancer cases making up-to 75% of all cases.[3] ROS1 protein, a transmembrane receptor protein with a specific tyrosine kinase activity found out to be acting as a growth, differentiation and proliferation factor coded by ROS1 gene (a proto-oncogene, also known as MCF3) on long arm of chromosome 6 (6q22.1) .[4] ROS1 gene genetic rearrangements have been found in numerous malignant tumors most frequently in non-small cell lung carcinoma (NSCLC), gastric adenocarcinomas, ovarian cancers, cholangio-carcinoma, inflammatory myofibroblastic tumor (IMT), angiosarcoma, colorectal malignancies and epitheloid hemangioendothelioma (EHE).[5] The use of tyrosine kinase inhibitors therapy in treating NSCLC have been studied

vastly in modern medical sciences. Many tyrosine kinase inhibitors such as Crizotinib are showing promising results as an effective therapy in patients with NSCLC, showing alteration in *ROS1* gene.[6]

Research and study of ROS1 gene rearrangements has given valuable and significant insight in the pathogenesis of different malignancies which also includes the breast carcinoma as highlighted in current study.

ROS1 can be tested using multiple technologies for positivity. Of these, fluorescence in situ hybridization (FISH) assays utilizing break-apart probe for the ROS1 gene is the most frequently utilized, gold standard and relied upon test for detection of this specific mutation. Recently, Next Generation Sequencing (NGS) is an emerging and accurate test for ROS1 detection. Reverse transcription polymerase chain reaction (RT-PCR) us another molecular technique for detection. Finally, immunohistochemistry is available which utilizes the detection of ROS1 protein by technique of immunohistochemistry instead of genetic alteration detection for ROS1. ROS1 gene rearrangement results in a detached or split of signal in the bulk of cases, or less frequently in absence of 5' probe signal in translocation of FIG1 to ROS1.[7,8]

Fluorescence in situ hybridization (FISH), next generation sequencing (NGS) and reverse transcriptase polymerase chain reaction (RT-PCR) assays are costly and complex laboratory investigations in most modern laboratories requiring specialized equipment and specific technical personnel expertise. Alternate available investigations such as immunohistochemistry may be performed in laboratories where such advanced molecular processes are not available and where financial and expert manpower resource are limiting factors. Immunohistochemistry has the advantage of rapid evaluation and interpretation by surgical pathologists or histopathologists in diagnostic pathology. As such, a ROS1 antibody (EP282 clone) has been developed which is now increasingly utilized to detect ROS1 mutated proteins in carcinomas most frequently NSCLC.[9,10]

Invasive breast carcinoma shows various pathogenetic progression pathways in its tumor progression. The tyrosine kinase progression pathway has been researched in breast cancers most frequently by Epidermal Growth Factor molecules such as, ErbB or HER2. Various HER2 targeted therapies have been used e.g., Trastuzumab, Margetximab, Pertuzumab and fam-trastuzumab. Of these, Trastuzumab was the first HER2 targeted therapy approved in the 1990's. Various TKIs such as Lapatinib, Neratinib, Pyrotinib and Tucatinib are in trial phase and have shown good results when used as monotherapy and in combination with chemotherapy. [11]

In current study we determined the expression of ROS1 protein by immunohistochemical method, in invasive breast carcinoma and studied its correlation (proportion, intensity and expression scores) with status of hormone receptors (ER and PR) and HER2 (another molecule of EGFR family).[12] Correlation between immunohistochemical markers might have an impact on invasive breast carcinoma, both in view of prognosis and treatment.

METHODOLOGY:

This was a cross-sectional study performed in Department of Histopathology at Armed Forces Institute of Pathology (AFIP) Rawalpindi from May to December 2022 after approval from ethics committee [FC-HSP20-17/READ-IRB/21/1279] of Armed Forces Institute of Pathology. A total of 137 formalin fixed paraffin embedded (FFPE) tissue of cases having invasive breast carcinoma of no special type and its subtypes were included. The World Health Organization (WHO) sample size calculator was used to calculate the sample size keeping a confidence level of 95%, margin of error (d) of 0.8 and anticipated population proportion (P) of 0.333, which was the proportion of patients with invasive breast carcinoma from Hameedi *et al*.[13]

All patients diagnosed with invasive breast carcinoma of breast with any histologic grade whether on incisional or excisional biopsy were included in the study. Patients who have received chemotherapy and/or radiation and had extramammary tumor or metastatic tumor were excluded. All patients' demographic data, tumor characteristics were confirmed at the time of sample receipt. Samples taken only as resection/lumpectomy/mastectomy (excisional) and trucut biopsy specimen (incisional) were examined. All cases were initially stained with hematoxylin and eosin stain for confirmation of diagnosis and tumor characteristics by two histopathologists independently. All confirmed cases included in study were then immunostained for ROS1, ER, PR and HER2 using Leica Bond III fully automated IHC staining system. ROS1, EP282, ER 6F11, PR 16 and HER2 antibody clones were used as per manufacturer's instructions. Cytoplasmic, membranous and nuclear staining were assessed for ROS1, HER2 and hormone receptors (estrogen and progesterone receptors), respectively. Allred scoring system was utilized for analysis of immunostained slides for expression of ER and PR by assessing proportion and staining intensity of tumor cells and calculating into scores for final result. HER2 expression was analyzed as per CAP/ASCO guidelines.[14] Immunohistochemical expression of ROS1 was assessed as a percentage of cells stained (proportion) and intensity of staining (cytoplasmic staining) as displayed in Table-I. Cases with >1% cytoplasmic staining with weak, moderate to strong intensity were considered positive while absence of staining or staining in <1% of tumor cells were considered as negative.

IBM Statistical Package for the Social Sciences version 25 is used for analysis of research data. Mean and standard deviation were calculated for quantitative variables. Percentage and frequency were used for qualitative variables

like gender, grade, immunoexpression of ER, PR, HER2 and ROS1 in invasive breast carcinoma. Qualitative variables were compared using the Chi square test and a *p*-value of =0.05 was considered statistically significant.

RESULTS:

This study was conducted on a sample size comprising blocks from 137 patients histologically diagnosed with invasive breast carcinoma (ductal). The mean age of the population was 50.85 ± 12.17 years. 131 (95.6%) patients were women and 6 (4.4%) were men. A total of 20 (14.60%) patients had tumor grade I lesions, while 73 (53.28%) and 44 (32.17%) had grade II and III lesions, respectively. For immunohistochemical expression of estrogen receptors 78 out of 137 cases (56.93%) were positive. Of these ER positive cases, 12/78 (15.38%) were of total Allred core of 8/8, 26/78 (33.33%) were of total Allred score of 7/8, 18/78 (23.08%) were Allred score 5/8, 13/78 (16.67%) were Allred score of 4/8 and 9/78 (11.54%) were of total score of 3/8. For immunohistochemical expression of PR 76 (55.47%) of total 137 cases were positive. Of them 14/76 (18.42%) were of total Allred core of 8/8, 22/76 (28.95%) were of total Allred score of 7/8, 8/76 (10.53%) were Allred score 5/8, 23/76 (30.26%) were Allred score of 4/8 and 9/76 (11.84%) were of score 3/8. 37 (27.01%) of the total 137 cases were positive for HER2 expression, 11 (8.03%) were equivocal and 89 (64.96%) were negative. 54 (39.42%) of all cases showed ROS1 expression (figure 1). Of them, 41/54 (75.92%) cases showed proportion score 1 while 13/54 (24.07%) were of score 2-3. In this study, ROS1 expression evaluated by total stained cell proportion and immunostaining intensity did not show significant statistical correlation with status of ER and PR (evaluated by total stained cell proportion and immunostaining intensity and overall expression calculated by Allred score) as *p* values were greater than 0.05, these cases. This statistical insignificant correlation is explained in Table 2 and 3.

Table I: Immunohistochemical expression of ROS1

Proportion		Intensity		ROS1 Expression
Cells stained	Score	Staining Intensity	Score	
0 %	0	No staining	0	Negative
1-25 %	1	Weak	1	Positive
26-100 %	2	Moderate to strong	2 or 3	

Figure 1: Invasive breast carcinoma, poorly differentiated (A) with immunohistochemical expression of ROS1 (B)

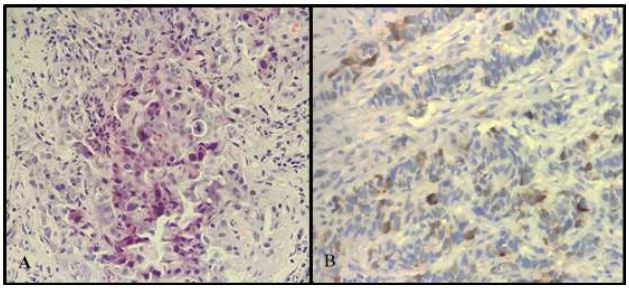

Table 2: Association between immunohistochemical expression of ROS1 expression, ER expression and PR expression (N=137).

Estrogen receptor expression association					
		ER Expression		Total n (%)	p-value
		Negative n (%)	Positive n (%)		
ROS1 Expression	Negative	30 (21.90%)	53 (38.69%)	83 (60.58%)	0.065
	Positive	29 (21.17%)	25 (18.25%)	54 (39.42%)	
Total		59 (43.07%)	78 (56.93%)	137 (100%)	
Progesterone receptor expression association					
		PR Expression		Total n (%)	p-value
		Negative n (%)	Positive n (%)		
ROS1 Expression	Negative	31 (22.63%)	52 (37.96%)	83 (60.58%)	0.064
	Positive	30 (21.90%)	24 (17.52%)	54 (39.42%)	
Total		61 (44.52%)	76 (55.47%)	137 (100%)	

The association between ROS1 expression and HER2 expression was noted to be statistically significant as p-value is <0.05 which is highlighted in Table 4. 23 (42.59%) out of 37 positively expressed cases for HER2 also expressed positive result for ROS1, while 7 (63.64%) equivocal out of 11 and 24 (26.97%) negative out of 89 cases expressed ROS1 immunohistochemically.

DISCUSSION

In this modern age of targeted therapy for carcinomas there is a need to look for most precise and specific therapies for effective treatment in these lethal conditions. ROS1, a receptor tyrosine kinase is usually associated with non-small cell lung carcinoma and in a large variety of other tumors.[15] Anti ROS1 drugs such as entrectinib, crizotinib and repotrectinib have been developed and used to target ROS1 gene translocation pathway in NSCLC.[16]

The important role of ROS1 targeted therapy in NSCLC inspired to study its role in many other human cancers such as spitzoid neoplasms, thyroid cancer, colorectal adenocarcinoma, glioblastoma multiforme, inflammatory myofibroblastic tumor, vascular tumors, angiosarcoma, atypical meningioma and other tumors.[17]

ROS1 mutation was first characterized by FISH assay and Tissue Microarray assays (TMA) both of which are expensive and technical procedures requiring specialized equipment and technical expertise. Recently antibodies against mutated ROS1 protein assessed by immunohistochemistry proved to be valuable, in NSCLC.[18]

Molecular classification of breast cancer based on expression or loss of hormonal receptor expression (ER & PR) and EGFR most commonly HER2 expression is one of the most significant aspects of decision making in treatment of invasive breast carcinoma. Immunohistochemical evaluation of ER, PR and HER2 in invasive breast cancer represent a critical

part in molecular classification and is a critical factor to be correlated with other tumor facets.[19, 20] In this research, we compared the immunohistochemical occurrence of ER, PR and HER2 (molecular classification markers) with immunohistochemical expression of ROS1 in invasive breast carcinoma (ductal). By this assessment the prognostic and predictive value of ROS1 in invasive breast carcinoma can be found.

In our research, statistically significant correlation was not found between immunoexpression of ROS1 and hormonal receptor status of estrogen receptor and progesterone receptor. Hormone receptor status was assessed by Allred scoring system as recommended by international guidelines. As per Allred score neither proportion score nor staining intensity correlated significantly with ROS1 histochemical expression (comprising tumor cells-stained proportion score and staining intensity score).

Eom M, *et al* in his study of occurrence of ROS1 protein expression in invasive breast carcinoma with histologic grade, ER status and HER2 status. In their study, ROS1 was expressed in 70% of ER positive cases and 30% of ER negative cases, thus ROS1 expression was significantly enhanced in ER positive cases. In their study ER expression and staining intensity were not correlated with ROS1 expression. ROS1 was positive in 70.9% HER2 negative and 29.1% of HER2 positive cases, which was not statistically significant.[21] In our study, 25 out of 54 positive ROS1 cases (46.30%) were also positive for ER, while 29 out of 54 (53.70 %) were negative for ER, while for PR expression 24 out of 54 ROS1 positive cases (44.44%) were positive for PR and 30 out of 54 (55.56%) were negative.

In our study, ROS1 was expressed in 23 of 37 (62.16%) HER2 positive cases while it was negative in 14 of 37 (37.84%) HER2 positive cases. Hence, ROS1 immunohistochemical expression was significantly correlated with HER2 expression (*p* value of <0.001).

In a somewhat similar study conducted by Hameedi *et al* immunohistochemical expression of ROS1 was correlated with ER, PR and HER2 expression. It was found that a statistically significant correlation was found among ROS1 expression and HER2 expression as 70 % of ROS1 expressive cases were also positive for HER2, while no significant correlation was found with ER and PR expression.[13]

Raut A *et al* concluded that ROS1 immunohistochemistry is not a true diagnostic and predictive screening test in breast carcinoma as none of the 631 patients with breast carcinomas demonstrated positive immunohistochemical staining for ROS1. However, it was significantly expressed in our study population. [22]

Li K *et al* studied genetic mutation profile of Chinese HER2

Table 3: Association between ROS1 proportion score, Estrogen receptor proportion score and Progesterone receptor proportion score (N=137)

Estrogen receptor proportion score association						
		ER Proportion Score			Total n (%)	p-value
		Negative n (%)	Score 1 to 3 n (%)	Score 4 to 5 n (%)		
ROS1 Proportion Score	Score 0	23 (16.79%)	31 (22.63%)	29 (21.17%)	83 (60.58%)	0.133
	Score 1	15 (10.95%)	18 (13.14%)	8 (5.84%)	41(29.93%)	
	Score 2-3	10 (7.30%)	1 (0.73%)	2 (1.46%)	13 (9.49%)	
Total		48 (35.04%)	50 (36.50%)	39 (28.47%)	137 (100%)	
Progesterone receptor proportion score association						
		PR Proportion Score			Total n (%)	p-value
		Negative n (%)	Score 1 to 3 n (%)	Score 4 to 5 n (%)		
ROS1 Proportion Score	Score 0	26 (18.98%)	30 (21.90%)	27 (19.71%)	83 (60.58%)	0.145
	Score 1	17 (12.41%)	19 (13.87%)	5 (3.65%)	41 (29.93%)	
	Score 2-3	10 (7.30%)	1 (0.73%)	2 (1.46%)	13 (9.49%)	
Total		53 (38.69%)	50 (36.50%)	34 (24.82%)	137 (100%)	

Table 4: Association between ROS1 expression and HER2 expression (N=137)

		HER2 Expression			Total n (%)	*p*-value
		Negative n (%)	Equivocal n (%)	Positive n (%)		
ROS1 Expression	Negative	65 (47.44%)	4 (2.92%)	14 (10.22%)	83 (60.58%)	<0.001
	Positive	24 (17.52%)	7 (5.11%)	23 (16.79%)	54 (39.42%)	
		89 (64.96%)	11 (8.03%)	37 (27.01%)	137 (100)	

positive patients in order to evaluate response of anti HER2 responses. In their study ROS1 mutation by NGS was found in 5 patients out of 40 belonging to HER2 positive group with a *p* value of 0.049.[23]

Eggmann H *et al* in his research established that HER2 overexpression is a poor prognostic indicator in breast cancer.[24] Hence, based on our study results of significant relation between HER2 and ROS1 expression, we can indicate that ROS1 immunohistochemical expression could represent a factor of poor prognosis in addition to HER2. Also supportive of this statement is the result of Force J *et al* establishing that ROS1 alterations were strongly associated with metastatic disease of the breast to CNS and lymphoid organs.[25]

Based on discoveries there is a need to study ROS1 expression in breast carcinoma in relation to prognostic significance and to follow up the patients for a significant period. Also ROS1 expression could be adopted for targeted therapy in invasive breast carcinoma by inducing growth inhibition and cell growth as highlighted by O'Neil SR. et al. [26] ROS1 immunohistochemical expressions need to be studied more in invasive breast carcinoma especially if there is an increased consideration of use of targeted therapy for TKIs in cases of breast carcinoma showing alteration of ROS1.

CONCLUSION

The study finding of significant number of ROS1 expressing cases in HER2 positive invasive breast carcinoma can be more revealing in the understanding of pathogenesis of breast carcinoma. In addition, it can also lead to use of certain recent tyrosine kinase inhibitors for treatment of this most common carcinoma in females.

Authors Contribution:
Muhammad Umair: Data collection, analysis, abstract writing and references
Ahmed Ahson Khan: Data analysis, interpretation and diagnosis
Nighat Jamal: Data collection and diagnosis
Akhter Ali Bajwa: Discussion and literature review
Tabish Hassan: Statistical analysis
Muhammad Umair Khan: Interpretation of results and conclusion

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