P-Glycoprotein Expression in Indian Breast Cancer Patients with Reference to Molecular Subtypes and Response to Anthracycline-Based Chemotherapy—a Prospective Clinical Study from a Developing Country

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Abstract

Chemo-resistance is an important factor determining the response of tumor to neoadjuvant chemotherapy (NACT). Our study was aimed to determine the role of P-glycoprotein (P-glyp) expression as a predictor of response to NACT in locally advanced breast cancer (LABC) patients with special reference to molecular subtypes. Sixty cases of locally advanced breast cancer (LABC) were subjected to trucut biopsy and the tissue samples were evaluated immunohistochemically for P-glyp, ER, PR, and Her 2 neu status. Pre- and post-NACT P-glyp expression was correlated with clinical response (using WHO criteria after three cycles of CEF regimen) and molecular subtypes. The change in the P-glyp expression before (pre-) and after (post-) NACT was statistically significant with higher stage ($p = 0.02$), hormonal negative molecular subtypes ($p = 0.01$), and poor clinical response ($p = 0.01$). Pre-NACT-positive P-glyp expression is associated with higher stage and hormonal negative molecular subtypes and poor clinical response. The increased expression of P-glyp induced by NACT likely explains the concept of acquired chemo-resistance and may prove as an intermediate checkpoint in determining chemo-sensitivity for further treatment so that additional doses of ineffective chemotherapy may be avoided in non-responders translating into better patient safety.

Keywords P-Glycoprotein · Breast neoplasm · Neoadjuvant chemotherapy · Clinical response · Molecular subtypes

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Introduction

Breast cancer is one of the leading causes of mortality worldwide with a much more dismal situation existing in developing countries [1]. Now being considered a systemic disease especially in advanced stages, multimodality treatment in the form of chemotherapy, radiotherapy, and targeted therapy in adjuvant surgery forms the basis of treatment in these patients. Neoadjuvant chemotherapy (NACT) has become an integral part of the management of breast cancer especially in developing countries like India where patients present in late stages with advanced disease [2–6]. Inter-patient chemo-response is variable due to several factors like absorption, distribution, metabolism, and elimination of drugs mediated by drug transporters and drug-metabolizing enzymes that constitute resistance at the cellular level in individuals [7]. One of the proposed mechanisms in literature is the expression of 170 K Daltons membrane glycoprotein better known as P-170 or p-glycoprotein (P-glyp) encoded by MDR-1 gene in humans [8–12].

Immunohistochemical (IHC) detection using monoclonal antibodies for P-glyp has been considered to be a more sensitive method of detection compared to electrophoresis studies [13–15]. Though the expression of P-glyp in pretreatment setting is linked to chemo-resistance in tumors like hematological malignancies, detecting its presence in breast cancer still remains a debate [16–23].

We have therefore investigated P-glyp expression in primary breast cancers by IHC using C219 mouse monoclonal antibody with special relation to the stage of the disease, molecular subtypes, and clinical response to NACT.

Materials and Methods

This prospective study was conducted between August 2013 and July 2014. Sixty breast cancer patients proven on FNAC with locally advanced disease as per UICC AJCC clinical staging were recruited after proper written and informed consent and ethical approval from institutional ethics committee vide no. 65 ECM II-C/P5.

Trucut biopsy was done to confirm the diagnosis in all patients and P-glyp expression was assessed from the tissue sample. Also, estrogen receptor (ER), progesterone receptor (PR), and Her 2 new receptor (Her 2 Neu) status were assessed from the core biopsy samples.

Routine investigations included complete blood counts, platelet counts, and renal and liver function tests. Diagnostic bilateral mammogram/ultrasound was done as necessary. Metastatic work up included chest X-ray, ultrasound abdomen and a bone scan of all patients. Chest or abdomen CT was done as necessary. A baseline ECG was done and cardiology clearance was sought in all patients as all patients received

epirubicin-based chemotherapy regimen. Detailed cardiac workup (echocardiography) was done on abnormal/suspicious clinical and/or ECG findings. Detailed history and examination were done in all patients. WHO clinical response criteria were used to assess response in all patients.

At the same time, P-glyp expression was again quantified in all patients by doing a second trucut biopsy. The tissue bits were taken and stored in formalin and assessed for P-glyp expression using the immunohistochemistry (IHC) technique (Fig. 1).

Methods of P-glyp Expression Using IHC Technique

The CD243/glycoprotein P monoclonal antibody (C219) from Thermo Scientific Pierce Antibodies is a mouse monoclonal antibody to C219 to ABCB1, ATP-binding cassette, sub-family B (MDR/TAP), member 1A, Abcb1a, Mdr1a. This antibody recognizes rat, mouse, hamster, and human antigen. The formalin-fixed paraffin extracted tissue blocks were sectioned at 3–4 μ m, using a microtome (Leica, Germany), mounted on tissue bond-coated slides (Biocare, USA). Immunohistochemistry was done by the standard protocol. The tissue section on coated slides was fixed overnight at 60 °C in a dry oven, deparaffinized in xylene and rehydrated through graded ethanol series. Sections were blocked with 3% hydrogen peroxide in methanol for 30 min to quench any endogenous peroxidase activity, if present, and were then processed for antigen retrieval in Pascal (DAKO Cytomation, California) by placing in sodium citrate buffer (pH 6.0). Sections were incubated for an hour with P-glycoprotein antibody (C219), followed by treatment with polymer-based secondary antibody kit (Dakopatts, Envision kit, Denmark). Bound antibody was visualized using diaminobenzidine. Sections were counter-stained with hematoxylin and mounted. Positive and negative (by omitting primary antibody) controls were run with all batches.

Measuring WHO Clinical Response

Clinical assessment of the tumor was done and measurements of the two longest diameters were taken using vernier calipers. Patients received three cycles of chemotherapy (CEF regimen—cyclophosphamide 500 mg/m²; epirubicin 100 mg/m²; 5-fluorouracil 500 mg/m²). Three weeks after the completion of the third cycle of chemotherapy, when patient visited for the fourth cycle, the response was assessed clinically.

Objective responders were defined as patients with complete response (CR) or partial response (PR). Non-responders included patients with the stable (SD) or progressive disease (PD).

Fig. 1 **a** Cells staining with P-glycoprotein (grade 2+). **b** Cells staining with P-glycoprotein (grade 3+)

Statistical Analysis

Statistical analysis was carried out using SPSS 17.0 software. The correlation analyses between the immuno-phenotypes and the various clinicopathological and biological factors were analyzed. Mean, median, range, and standard deviation was calculated. Unpaired *t* test, Mann-Whitney *U* test, Kruskal-Wallis, Kendal's tau *b* test was used for categorical data and *p* values < 0.05 were considered significant.

Results

Sixty patients were recruited initially. Eleven patients did not complete the treatment protocol and both tract biopsies could not be performed in these patients, so were excluded from the final analysis.

Of these, 38 patients were postmenopausal with a mean age of 52.4 years and 11 were premenopausal with a mean age of 35.3 years (range 21–72 yrs.). It was found 7.5% of breast cancer patients had T2 breast cancer, 70.1% had T3 breast cancer, and the remaining 22.4% of patients showed involvement of the chest wall and/or skin (T4 disease). 26.9% patients had no clinically detectable lymph nodes, 47.8% had N1 nodal status, 23.9% of the patients had N2 nodal status, and only 1.5% of the breast cancer patients had N3 nodal status. 86.6% of breast cancer patients showed no metastasis and remaining 13.4% patients had evidence of metastasis.

The majority (33/49) of the patients in LABC were stage IIIB. On comparing relationship of the stage with the expression of P-glyp, it was observed that increased number of patients stained positive for P-glyp post NACT especially in higher stages (IIIB/C and IV versus II) (Table 1, *p* = 0.02).

Thirty-five patients who were included in the study belonged to hormonal negative molecular subtype (triple negative—14; Her 2 new enriched—21). P-glyp expression was significantly increased in these patients post NACT compared

to hormonal positive molecular subtypes (Luminal A and B) (Table 2, *p* = 0.01).

The clinical response was assessed using WHO clinical criteria. Twenty-five patients were responders (complete response in 3 and partial response in 22 patients) while the rest 24 patients were non-responders. Regarding the relationship of the levels of staining of P-glyp in breast cancer cells when compared with the response to chemotherapy, it was observed that increased number of patients stained positive for P-glyp after receiving chemotherapy, more so in patients showing a non-response. Comparing statistically this increase in P-glyp expression in non-responders was found to be significant (Table 3, *p* = 0.01).

The change in the P-glyp expression before and after NACT was statistically significant with higher stage, hormonal negative molecular subtypes, and poor clinical response.

Discussion

Breast cancer is the most common malignancy in India after cervical cancer with most of the patients presenting in late

Table 1 Relationship of staining of P-glycoprotein with stage from pre-chemotherapy to post-chemotherapy

Stage	Staining							
	Pre-chemotherapy				Post-chemotherapy			
	Nil	Present			Nil	Present		
	No.	%	No.	%	No.	%	No.	%
II	8	20.0	0	0.0	8	40.0	0	0.0
III	26	65.0	7	77.8	9	45.0	24	82.8
IV	6	15.0	2	22.2	3	15.0	5	17.2
Total	40	100.0	9	100.0	20	100.0	29	100.0

Kendal's tau *b* test *p* = 0.02 (Significant)

Table 2 Relationship of staining with molecular subtypes of breast cancer from pre-chemotherapy to post-chemotherapy

Luminal	Staining							
	Pre-chemotherapy				Post-chemotherapy			
	Nil		Present		Nil		Present	
	No.	%	No.	%	No.	%	No.	%
A	5	12.5	0	0.0	4	20.0	1	3.4
B	9	22.5	0	0.0	9	45.0	0	0.0
Her 2 neu enriched	16	40.0	5	55.6	6	30.0	15	51.7
Triple Negative	10	25.0	4	44.4	1	5.0	13	44.8
Total	40	100.0	9	100.0	20	100.0	29	100.0

Kendal's tau *b* test $p = 0.01$ (significant)

stages. Multiple factors can be attributed to this treatment delay like rampant illiteracy, poor reach to expert advice, and aggressive disease in Indian women [1–5]. The recommended approach for the ideal management of LABC is multimodality, multidisciplinary approach with intent to provide local as well as systemic control of the disease [1–6]. Considering the Gompertzian growth curve for neoplastic diseases, the undetectable systemic micro-metastases at diagnosis have led to systemic treatment now to be considered equal and important to local surgical control [22–25]. Also, surgery does not alter the incidence of distant failure in these patients as they often have micro-metastases at the time of disease presentation. With the advent of NACT in the late 1970s with a high objective response rate, it has been proven to be an effective systemic control to convert unresectable tumors to smaller tumors making them more amenable to local therapy in the form of surgical resection and/or radiotherapy. It is generally presumed that there is a positive correlation between the tumor response and prognosis of the disease [3, 20, 21]. The added advantage of NACT is that it provides an in vivo

Table 3 Relationship of staining of P-glycoprotein with response from pre-chemotherapy to post-chemotherapy

Response	Staining							
	Pre-chemotherapy				Post-chemotherapy			
	Nil		Present		Nil		Present	
	No.	%	No.	%	No.	%	No.	%
Complete response	3	7.5	0	0.0	3	15.0	0	0.0
Partial response	22	55.0	0	0.0	15	75.0	7	24.1
Progressive disease	9	22.5	6	66.7	2	10.0	13	44.8
Stable	6	15.0	3	33.3	0	0.0	9	31.0
Total	40	100.0	9	100.0	20	100.0	29	100.0

Kendal's tau *b* test $p = 0.01$ (significant)

response check for assessment of tumor response from which prognostic influence can be drawn and future adjuvant treatment can be decided.

One of the major problems in the treatment of cancer has been the development of resistance to cytotoxic drugs and an ideal predictor still remains elusive [3]. One of the most researched and documented mechanism is multidrug resistance and is characterized by cross-resistance to a variety of structurally unrelated drugs post exposure to only one of them, most commonly documented from anthracyclines, vinca alkaloids, dactinomycin, and paclitaxel [26]. The MDR gene, *mdr1* (also known as ABCD1), that encodes for a transmembrane protein of 170 K Daltons, often termed as P-glycoprotein is present in many MDR cell lines and is believed to act as an energy-dependent drug efflux pump [8, 13]. Human *mdr1* expression in a variety of normal tissues and tumors has been studied by measurement of *mdr1*-mRNA including breast cancer. However, the detection of mRNA in a homogenized tumor sample does not convey about the cell expressing *mdr1* and its distribution in the cells in the tumor. Besides, this technique may not be sensitive to detect a minute population of MDR cells. Thus, the electrophoretic methods such as Northern blotting and Western blotting in detecting P-glyp expression within tumor samples is not of choice. With the introduction of monoclonal antibodies to P-glyp and immunohistochemistry technique, these problems have been effectively taken care of and offered a greater sensitivity and specificity in detection compared to electrophoretic methods [13, 15].

The P-glyp expression has been reported in the literature to be present in several normal human tissues also and indicates a possible role to protect cells against xenobiotics and endogenous cellular toxins [27–30]. High levels of P-glyp has been linked to intrinsic chemo-resistance to tumors like kidneys, colon, adrenals, leukemias, cervical cancer, and soft tissue sarcomas [12, 29]. However, the role of P-glyp expression exclusively in breast cancer remains an enigma with most of the published data suggesting that its expression in naive breast cancer is not a usual phenomenon. Monoclonal antibodies have already been used to detect P-glyp in several human cancers. C219, which reacts with an internal epitope of P-glyp, has revealed P-glyp expression in hematologic as well as solid organ malignancies. Besides, C219 has confirmed P-glyp expression in both untreated and treated breast cancers [13]. Sugawara et al. detected one positive case out of nine tumor samples using another MRK 16 monoclonal antibody, which reacts with an external epitope of P-glyp [19]. In the present study, C219 antibody was used and observations confirmed the presence of P-glyp expression in primary untreated breast cancer though in minute percentage (9/49 patients). Although reported in the literature that expression in primary breast cancer is not a common occurrence, few anecdotal reports have reported the extraordinary high incidence of P-glyp in untreated breast cancer [21, 22].

The reported response rates following NACT vary between 49 and 94% in published literature and this has been due to different chemotherapy combinations are given at variable doses and intervals [3]. The response rate in our study was 51% (25/49 patients were responders). The overall clinical response was observed to be on the lower side due to smaller sample size, late presentation with the advanced locoregional disease in the majority or a combination of both. Ro et al. have reported that intrinsic resistance may play a role in the failure of induction chemotherapy in LABC [22]. In the present study, 9/49 (18.4%) patients were P-glyp positive in the pretreatment group and the number increased to 29/49 (59.2%) when the results were reassessed after three cycles of NACT. None of the patients who showed CR developed P-glyp positivity. Overall, 25/49 (51%) patients were clinical responders to NACT, out of which 7 patients developed P-glyp positivity post NACT. All of these seven patients only registered a partial response to systemic treatment. Nine patients who showed pretreatment P-glyp positivity fared poorly on NACT as “non-responders.” With the increase in P-glyp expression, the response rate was therefore found to drop. Trock et al. conducted a critical review and meta-analysis of 31 studies and found that patients with tumors expressing P-glyp were three times more likely to fail to respond to chemotherapy than patients whose tumors were P-glyp negative. P-glyp expression in breast tumors is associated with a poor response to chemotherapy [31]. There are only a few studies reporting an increase in the frequency of P-glyp expression during or after NACT [19, 20]. In the present study, P-glyp expression increased from 18 to 59% during NACT. This unexpected rise in the positivity rate is a valid explanation for the acquired chemo-resistance—a phenomenon that reestablishes itself here in breast cancer also. Therefore, immunohistochemical detection of P-glyp may be used to predict the non-response in breast cancer post NACT and could very well act as an intermediate check point in establishing drug sensitivity for further continuation of the chemotherapy regimen.

Reports establishing a relationship of hormonal receptors (ER, PR) with P-glyp expression have been published but the concepts of this linkage still remains elusive [32, 33]. To best of our knowledge this study for the first time reports the relation of P-glyp and molecular subtypes in breast cancer. Pretreatment positivity when categorized according to molecular subtypes, it was present only in hormonal negative breast cancer (Her 2 neu and triple negative subtypes). Only one patient with Luminal A subtype showed P-glyp expression in post-NACT testing. Meanwhile, the P-glyp positivity increased significantly in hormonal negative subtypes post NACT ($p = 0.01$).

The study highlights the importance of P-glyp expression in accessing response to NACT, particularly in non-

responders. Patients with positive P-glyp expression in pre-NACT stage are usually associated with a poor clinical response. Pre-NACT-positive P-glyp expression is associated with higher stage and hormonal negative molecular subtypes and poor clinical response however the association between these factors cannot be established in our study. The increased expression of P-glyp induced by NACT likely explains the concept of acquired chemo-resistance and may prove as an intermediate checkpoint in determining chemo-sensitivity for further treatment so that additional doses of ineffective chemotherapy may be avoided in non-responders translating into better patient safety.

Author Contribution MM contributed in the literature search, data acquisition, data analysis, and manuscript preparation and manuscript editing.

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SK contributed in literature search, data acquisition and manuscript preparation.

NH contributed in the data analysis, manuscript editing and review.

AAS contributed in the concept, design, and definition of intellectual content, manuscript editing and review.

Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

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