Improving accuracy of breast cancer biomarker testing in India

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There is a global mandate even in countries with low resources to improve the accuracy of testing biomarkers in breast cancer viz. oestrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2neu) given their critical impact in the management of patients. The steps taken include compulsory participation in an external quality assurance (EQA) programme, centralized testing, and regular performance audits for laboratories. This review addresses the status of ER/PR and HER2neu testing in India and possible reasons for the delay in development of guidelines and mandate for testing in the country. The chief cause of erroneous ER and PR testing in India continues to be easily correctable issues such as fixation and antigen retrieval, while for HER2neu testing, it is the use of low-cost non-validated antibodies and interpretative errors. These deficiencies can however, be rectified by (i) distributing the accountability and responsibility to surgeons and oncologist, (ii) certification of centres for testing in oncology, and (iii) initiation of a national EQA system (EQAS) programme that will help with economical solutions and identifying the centres of excellence and instill a system for reprimand of poorly performing laboratories.

Key words Biomarker - breast - cancer - HER2neu - oestrogen receptor - progesterone receptor - quality assurance

Introduction

The explosion of theranostics (a portmanteau of therapeutics and diagnostics) has led to an emergence of immunohistochemistry (IHC)-based predictive markers that are used to treat patients. The three biomarkers viz. oestrogen receptor/progesterone receptor (ER/PR) and HER2neu have markedly improved the prognosis of breast cancer with the use of appropriately targeted therapy, leading to the mandate of compulsory testing in all breast cancers and even in recurrences1-3. The emergence of additional therapies beyond tamoxifen and second-generation anti-HER2neu inhibitors has further intensified the interest in improving the accuracy of testing for these biomarkers4. IHC-based predictive markers are popular as these are cheaper, easy to implement and serve as a first screen to look for targets e.g. in breast cancer, only the equivocal (score 2) samples are reflex tested by fluorescent in situ hybridization (FISH) to confirm HER2neu amplification reducing the burden on testing laboratories. However, IHC has its unique set of flaws and the errors produced are frequently labelled as the black box of IHC testing5,6.

From the experience of large clinical trials, it is evident that there is a great variability in testing biomarkers in breast carcinoma which has prompted the American Society of Clinical Oncology (ASCO) and College of American Pathologists
(CAP) (ASCO-CAP), National Comprehensive Cancer Network (NCCN) and individual countries (Spain, Sweden, Australia, Austria, etc.)\textsuperscript{13,7,11} to come out with the guidelines to ensure accurate testing. Following these guidelines is mandatory for testing laboratories in the most developed countries. In low-resource countries, though the guidelines for reporting are followed, the testing methodology is fallacious and cost is often cited as the chief cause for delay in implementation of good practices. This review addresses the present state of testing for ER/PR and HER2neu biomarkers (global versus India) and steps that are required to improve on it.

**Learning from global experience**

Globally, hormone receptor-positive cancers are the most common subtype of breast cancer, accounting for 78-80 per cent of all cases\textsuperscript{1,3,7,12,13}. The global HER2neu IHC-based positivity rates range from 11 to 20 per cent\textsuperscript{23,14,15}. The problem of erroneous results of ER/PR and HER2neu testing is universal and not confined to countries with low resources\textsuperscript{5}. In a review of pathology testing procedures of patients enrolled in the ‘Breast International Group’ (BIG) I-98 trial, 73 of 105 (69%) ER-negative tumours were found to have more than 10 per cent positive cells and 66 of 6100 (1%) tumours locally reported ER positive were found to have no staining\textsuperscript{16}. In an external quality assurance (EQA) programme involving 105 laboratories in Europe, reliable assays for ER and PR were found only in 24 (36\%) of 66 laboratories participating in the continual EQA in spite of all centres having clinically validated assays\textsuperscript{17}. In the Adjuvant Lapatinib and/or Trastuzumab Treatment Optimization trial, 4.3 per cent of tumours tested ER positive in local laboratories were found to be negative on central testing. More than 20 per cent of tumours were falsely reported as ER negative\textsuperscript{18}.

Approximately 20 per cent of HER2neu assays performed at the primary treatment site’s pathology department were incorrect when re-evaluated in a high-volume, central laboratory\textsuperscript{19,21}. A false-negative diagnosis will deny potentially life-extending therapy to a truly HER2-positive patient. On the other hand, a false positive will result in exposure to a drug that has significant cardiotoxicity and exorbitant drug cost. High discordance rates between IHC and FISH are due to technical issues and should not be used to condemn the technique itself\textsuperscript{11}. While the superiority of one method vs the other remains controversial, screening all cases with IHC and triaging selected cases for FISH testing is acceptable\textsuperscript{22,23}.

The need for constant monitoring and EQA programme was felt two decades ago, and the United Kingdom National External Quality Assurance programme (UKNEQAS) was established nearly 30 yr ago (http://www.ukneqasiccish.org). UKNEQAS has published some seminal articles on improving ER and PR testing accuracy across the globe\textsuperscript{17,24,25}. Proficiency testing for ER, PR and HER2neu was developed by the Royal College of Pathologists of Australasia Quality Assurance Program in 2001, and an audit on more than 8000 patients indicated that though overall the results for ER, PR and HER2neu fell within the established parameters, a number of individual laboratories did not meet the target values and the variation in results impacted patient treatment decisions\textsuperscript{19}. In the widely known ‘Canadian disaster’, a false-negative index ER test report in Eastern Health led to investigation of the accuracy of ER testing in Newfoundland and Labrador and 40 per cent of over 2000 originally ER-negative cases were found to be ER positive on re-testing\textsuperscript{26}. Although Canada has a national health care system, health care delivery is handled regionally, and Newfoundland had no regulatory body for accreditation or setting standards for conduct of clinical laboratory tests\textsuperscript{26}. Within Canada, strong public reaction to the above event motivated a push for systemic changes in medical training and laboratory staffing\textsuperscript{26}. Programme to regularly monitor ER/PR and HER2neu data from provinces was set up, and ER or PR positivity rates were monitored in several provinces\textsuperscript{27}. The Canadian IHC Quality Control Programme subsequently evaluated 31 participating laboratories for ER/PR in 44 breast carcinomas and reported 100 per cent agreement when indeterminate results were excluded\textsuperscript{28}. The Nordic Immunohistochemical Quality Control (NordiQC) documented that 20 per cent of the staining results in breast cancer IHC module were insufficient for diagnostic use\textsuperscript{29}. Some of these EQA system (EQAS) programmes exercise more control and have a mandate for regulatory action against defaulting laboratories, e.g. UKNEQAS is required to notify the National Quality Assurance Advisory Panel of any cases of persistent poor performance in participating UK clinical laboratories (http://www.ukneqasiccish.org).

Even in the low-resource countries, steps are being taken to improve ER/PR and HER2neu testing. If the positivity rates are the judging ruler, one study from Africa documented an ER positivity of 72.8 per cent,
PR in 64.8 and 17.6 per cent HER2 positivity similar to that observed in western countries\textsuperscript{10}. The low per cent positivity in Asian countries may be at least in part due to testing issues. However, several studies from Philippines, Bangladesh, Vietnam and Malaysia have reported that with improved fixation and testing practices, the incidence of ER/PR is between 60 and 70 per cent\textsuperscript{13}. In a study from the National Cancer Center in China, the ER/PR positivity rate was 78.4 and 79.7 per cent, respectively, while 25.5 per cent were HER2 positive\textsuperscript{32}.

Recognizing the lacunae in testing methodology, Scientific Partnership for HER2 Testing Excellence (SPHERE) training programme (sponsored by Roche Pharma) was initiated in the Asia-Pacific region including 70 countries and supporting 120 laboratories for the UKNEQAS IHC and \textit{in situ} hybridization (ISH) EQA programme\textsuperscript{33}. A jump in the ‘pass rates’ for UKNEQAS programme, from 39 to 61 per cent was seen in HER2neu run across these countries (Dr Ibrahim Merdol, personal communication).

**The Indian scene on breast cancer biomarker testing**

One way of ensuring uniformity is to have data on the incidence of these biomarkers (ER/PR and HER2neu) for India to establish the minimum and maximum cut-offs. Data on biomarker prevalence in India are however, variable chiefly due to test-related issues. Most studies that reported lower hormone receptor positivity in patient population justified that our patient population was a decade younger than Western countries and had higher grade of tumours\textsuperscript{34,35}. A summary of all studies published in this regard is given in Table including the results of our laboratory. The most valid hormone receptor positivity in Indian patient population reached between 60 and 70 per cent while rates for HER2neu positivity in breast cancer were between 20 and 26 per cent, thus being close to the global rates\textsuperscript{36-50}. An eight-year audit from our institute using manual testing for ER and PR documented the highest rate of 56 per cent\textsuperscript{37}. However, a six year analysis from 2009 to 2014 of 8270 patients revealed a hormone receptor positivity rate of up to 70 per cent (unpublished audit results) (Table). While anti-HER2neu drug herceptin arrived on horizon years ago for treating patients, laboratory guidelines in India have not been evolved. As there is no health insurance in place and patients pay for these tests in most institutes, there is a tendency for laboratories to economize. While the repertoire of antibodies available for ER/PR is limited, a bevy of antibodies are available in HER2neu testing. Due to high cost involved with the testing, most laboratories in India do non-FDA-approved/homebrew assays. As per the ASCO guidelines\textsuperscript{2}, a laboratory is certified for HER2neu testing if the concordance rates

| Author               | Total patients | ER positive/PR positive (%) | HER2neu positive (%) |
|----------------------|----------------|-----------------------------|----------------------|
| Desai \textit{et al}\textsuperscript{36} | 798            | 46.1                        | -                    |
| Shet \textit{et al}\textsuperscript{37} | 11,780         | 57                          | -                    |
| Zubeda \textit{et al}\textsuperscript{38} | 619            | 32.56                       | 36.71                |
| Doval \textit{et al}\textsuperscript{39} | 1284           | 63.4                        | 23                   |
| Patnayak \textit{et al}\textsuperscript{40} | 389            | 61                          | 29.6                 |
| Ghosh \textit{et al}\textsuperscript{41} | 2001           | 51.2                        | 16.7                 |
| Munjal \textit{et al}\textsuperscript{42} | 107            | 41.1                        | 29                   |
| Vaidyanathan \textit{et al}\textsuperscript{43} | 368            | -                           | 43.2                 |
| Rao \textit{et al}\textsuperscript{44} | 126            | 36.5                        | 3 cases positive     |
| Singh \textit{et al}\textsuperscript{45} | 206            | 44                          | 34.2                 |
| Manjunath \textit{et al}\textsuperscript{46} | 250            | 49.2                        | -                    |
| Kumar \textit{et al}\textsuperscript{47} | 112            | 42                          | 46.37                |
| Ambroise \textit{et al}\textsuperscript{48} | 321            | 59                          | 27                   |
| Tata memorial hospital data (2009-2014)\textsuperscript{49} | 8270           | 70                          | -                    |
| Tata memorial hospital (2012)\textsuperscript{50} | 4269           | -                           | 26.1                 |

\textsuperscript{1}Unpublished data
are greater than 90 per cent for score 3+ and FISH amplified cases while only 1-5 per cent of 0/1+ are FISH amplified. However, a trend to play safe and give a high equivocal or score 2+ results to avoid false negatives or false positives has been observed, beating the purpose of IHC for HER2neu (unpublished observations). Furthermore, the primary cancer health care in breast cancer is often rendered by a physician or surgeon without any specific training in oncology. Hence, the patient often ends up with a specimen that is poorly fixed and not fit for evaluation, putting pressure on the referral cancer testing laboratories for ensuring test accuracy.

Fig. 1. A patient affected by gap in biomarker testing (A) haematoxylin and eosin section confirmed that tumour was poorly fixed (H & E: 200×) (B) weak HER2neu staining due to poor fixation (immunoperoxidase: 200×), (C) higher power to indicate that HER2neu would be interpreted as score 1+/negative (immunoperoxidase: 400×), and (D) tumour as tested by fluorescent in situ hybridization found to be HER2neu amplified (Oil).

Pre-analytical factors

Improper fixation of tissue is the single most factor resulting in non-standardized results across low-resource countries where the site of specimen generation and testing laboratory are often differently located. Tissue specimens that are refrigerated or fixed in inadequate formalin are more likely to undergo autolysis and loose ER activity.

In the UKNEQAS, a study of 25 tumours showed that a delay of up to 120 min in fixation resulted in reduction in ER immunopositivity. Specimen are often refrigerated to delay loss of antigenicity; however, in a study of the 25 refrigerated samples, eight (32%), six (24%) and six (24%) cases showed reduction for ER, PR and HER2neu expression, respectively, in spite of refrigeration. Khoury et al. reported that overnight storage at 4°C resulted in loss of tissue antigenicity that was similar to leaving specimen without fixation for eight hours and recommended immediate specimen delivery rather than refrigeration. Specimens operated for breast cancer late in the week are more likely to be fixed later and hence more ER/PR negative than specimens obtained on other weekdays. There is a strong contention for stopping PR testing in breast cancer to economize. However, PR is a robust antigen less affected by fixation-related issues and often ensures hormonal therapy in a patient who is reported as ER negative falsely due to pre-analytical issues such as poor fixation. An eight-year audit of 11,000 odd cases at our institute revealed that improved fixation resulted in reduction in breast cancers that expressed only PR from 20 to three per cent because the improved fixation resulted in increased demonstration of ER and the category of tumours that expressed both ER/PR expanded. ER-positive and PR-negative tumours are also less responsive to endocrine therapy (particularly tamoxifen) as opposed to ER-positive and PR-positive tumours helping prognostication. PR negativity can also influence the therapeutic decision to offer adjuvant chemotherapy in addition to adjuvant endocrine therapy in selected patients. Given the superior test results in core biopsy which are rapidly and better fixed, most developed countries perform testing for ER/PR and HER2neu on core biopsy. However, core biopsies for primary diagnosis of breast cancer are not possible at all the places in resource-poor countries.

Analytical variables

The most common causes for discrepancy in analytical methods are non-validated antibodies,
poorly calibrated ready-to-use products, insufficiently calibrated antibody dilutions (20%), insufficient or erroneous epitope retrieval (27%), less sensitive visualization systems (19%) and stainer platform-dependant protocol issues.

The main advantage of automation in IHC is better-standardized retrieval and staining protocols. However, it is a dual-edged sword, and besides increasing costs, it separates the staff from the staining process increasing the likelihood of insufficient knowledge to handle troubleshooting issues. As compared to manual staining automated immunostaining results in increased specificity, increased positive predictive value and increased efficiency of HER2neu test results. Antigen retrieval with pressure cookers (besides fixation) was a major factor improving ER testing by manual staining in our laboratory. Tissues fixed for less than six hours and with lesser antigen retrieval time (<25 min) had poor staining scores in one study. How to select the best antibody for a specific antigen is complex but is aided by comparisons with a ‘gold standard’ and use of EQA data available from the UKNEQAS website (http://www.ukneqasiccish.org).

Several studies have reported their experience with different clones and companies for ER, PR and HER2neu antibodies. A study using standardized quantitative immunofluorescent ER assay demonstrated that SP1 clone for ER was at least eight per cent more sensitive and correlated better with patients’ outcome than 1D5 clone. Significantly higher PR values were obtained when the tumours were analyzed by the Ventana 1E2 RTU kit compared to the PharmDX kit (clone PR 1294). The staining results with 4B5 antibody for HER2neu indicated that it had a more robust performance than CB11 clone and perfect correlation with FISH is excellently reproducible. A FISH and IHC comparison study at our institute with Immunotech antibody A revealed that 66.6 per cent of score 2+ cases showed amplification on FISH due to antibody-related issues. Our observations (unpublished) with validated antibodies showed that only a quarter of our HER2neu equivocal cases were amplified. Results of validation and specificity of antibodies vary across the globe. For example, in UKNEQAS, 55-77 per cent of centres using 6F11 had satisfactory performance compared with only 35 per cent centres using 1D5, while another study documented that SP1 was a better antibody than 1D5. In an UKNEQAS study, while the laboratories using the DAKO Hercep test had the highest level of reproducibility in assay sensitivity and evaluation, the significant improvement in results by laboratories using other antibodies in the second assessment run suggested that stringent quality control and an ongoing quality assurance programme had the potential to improve the reliability of immunohistochemical assays for HER2neu, regardless of the brand of antibody used. Though getting global uniformity in these analytical variables especially antibody clone seems difficult, getting systems in place and participation in an EQAS ensures minimal variability.

Post-analytical interpretative error

The interpretative error for hormone receptor reporting may be less as the cut-off value is small (1%) Interpretative error is the most important factor yielding variable accuracy rates for HER2neu testing. In one study, the overall concordance between observers for equivocal HER2neu results was low (55.8%) but for negative and positive results it was very high. A unique trend of reporting was observed; pathologist with 100 per cent IHC and FISH concordance, usually had a tendency to play safe and reported a high number of equivocal cases while pathologists who reported clear cut results (positive or negative) had lesser concordance with FISH. We observed that pathologist reporting both HER2neu IHC and FISH at our institute had better concordance with the FISH results. Tumour heterogeneity is another cause for a false negative or positive report, and hence, it is
always better to compare the tissue in core biopsy with excision specimen. PR is more heterogeneous than ER and may produce discordant results in core biopsy. Carcinomas with HER2neu genetic heterogeneity can still have an overall negative HER2neu amplification status, despite still containing a significant number of tumour cells with HER2neu staining/score 3+ on IHC; hence, HER2neu heterogeneity should indicate need for FISH confirmation.

**Solutions to reduce the gap in ER/PR and HER2neu testing in India**

**Awareness and accountability**

As the ASCO-CAP guidelines have highlighted, the good pathology practice starts in the operation theatre. Surgeons should take additional responsibility of ensuring prompt transport of specimens or ensuring adequate fixation before these are dispatched to histopathology laboratory. Institutes should invest in training programmes for pathologist and technicians involved in an oncology service. There should be attempt at certification of laboratories before they sign out oncology and critical reports.

**American Society of Clinical Oncology-College of American Pathologists (ASCO-CAP) guidelines**

As per the ASCO-CAP 2007 HER2neu testing guidelines, samples where pre-analytical variables are unknown, had to be rejected, but this would result in most samples in low-resource countries as inappropriate for testing. Mandatory participation in external proficiency testing programme with at least two testing events (mailings) per year is essential in ASCO-CAP guidelines, making it an expensive mandate for most laboratories. These guidelines on the other hand, form a framework for use by all pathologists across the world to ensure uniformity. Most laboratories can also quote these guidelines to request for resources involved in an oncology service. There should be attempt at certification of laboratories before they sign out oncology and critical reports.

**Centralized testing**

UKNEQAS documented that when histological material from different sources were collected centrally and subjected to a common protocol of antigen retrieval using automated immunohistochemical analysis and assessment, uniform results for ER/PR were obtained. Furthermore, as HER2neu testing is considered a high-complexity test under the Clinical Laboratory Improvement Amendments of 1988, it must be internally validated to ensure accuracy and reproducibility before being offered by a laboratory. Various countries have come up with the national guidelines to ensure that testing performed in a cost-effective manner and accurately and hence define the minimum number of test to be done, e.g. 100 in situ hybridization (ISH) tests (two per week) be performed in each centre per annum to qualify for reporting HER2neu ISH. The fact remains that if pre-analytical conditions are below optimum, the use of a single laboratory and a standardized and automated staining method for ER/PR testing are not sufficient to reduce variability in ER/PR test results.

**Internal laboratory validation programme or audits**

Regular and ongoing audit of ER/PR and HER2neu testing should be undertaken to monitor test variability. The standardized results could fail in some situations, e.g. batch-to-batch variations in antibody. Laboratories should audit their overall annual negative, equivocal and positive rate for HER2neu using a combination of IHC and ISH or compare test results with another laboratory. The CAP study revealed a tendency to follow ASCO-CAP guidelines, but there were several lacunae. Of the laboratories comparing IHC HER2neu assays with an IHC test performed in another laboratory, only 56 per cent of laboratories used a recommended minimum of 25 cases.

**External quality assurance system (EQAS)**

EQAS can provide guidance on how to achieve the best IHC standards and participation in such programmes helps laboratories detect problems not identified by internal quality control. NordiQC reported that in the 14 runs of ER during 2003-2015, the proportion of sufficient stains increased from 45 per cent in the first run to about 70-90 per cent in the later runs. In line with this observation, the pass rate for ‘old’ participants was consistently higher than for the new ones in the latest run 73 versus 51 per cent. A web-based quality improvement training and a comparative study of accuracy of IHC tests of breast cancer biomarkers between a well-established laboratory in the United States and a field laboratory in Ibadan, Nigeria, demonstrated that this could be a useful and cost-effective tool for quality assurance of IHC and provide much-needed capacity building in resource-poor countries.

In India while most of institutes have quality systems in place, it is driven by economics. Though
the Indian Council of Medical Research (ICMR) has brought out a consensus document for management of breast cancer, there is presently no forum that can bring these heterogeneous practices on one platform and form an India-based guidelines. The National Cancer Grid (NCG) funded by the Government of India through the Department of Atomic Energy was formed in August 2012 with the mandate of linking 69 cancer centres across India. Forums like the NCG should initiate EQAS to establish guidelines that are implementable across the country. There should be a government mandate for all laboratories doing predictive marker testing to register with one of the forums. Once registered, the results of the participant laboratory should be monitored by a central laboratory annually and biannually. There is a need to divide the country into four zones (North, South, West and East) with centres of excellence in each zone covering all the testing laboratories in the region (Fig. 3).

Conclusion

There is a global initiative to recognize and rectify some of the loopholes in testing for ER/PR and HER2neu even in the low-resource countries. The variability in testing for these markers is rather wide in our country as compared to the other Asian nations and needs to be closed with an urgent national mandate by national bodies such as ICMR/NCG and by a team of professionals that encompass both government and health officials besides oncologists, surgeons and pathologists. Only then, one can ensure cost-effective and safe oncology care to the breast cancer patients.

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Conflicts of Interest: The author is on Roche Advisory board for biomarker testing in India and member of the SPHERE programme.

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