The quest for biomarkers: Computational exploration of the cancer genome

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CONCORDANCE OF CLINICAL AND MOLECULAR BREAST CANCER SUBTYPING IN THE CONTEXT OF PREOPERATIVE CHEMOTHERAPY RESPONSE

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MOLECULAR SUBTYPING OF BREAST CANCER: READY TO USE?

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ABSTRACT

ER, PR and HER2 status in breast cancer are important markers for the selection of drug therapy. By immunohistochemistry (IHC), three major breast cancer subtypes can be distinguished: Triple negative (TN_{IHC}), $HER2+_{IHC}$ and $Luminal_{IHC}$ (ER+IHC/HER2-IHC). By using the intrinsic gene set defined by Hu et al. five molecular subtypes ($Basal_{mRNA}$, $HER2+_{mRNA}$, Luminal A_{mRNA}, Luminal B_{mRNA} and $Normal\text{-}like_{mRNA}$) can be defined. We studied the concordance between analogous subtypes and their prediction of response to neoadjuvant chemotherapy. We classified 195 breast tumors by both IHC and mRNA expression analysis of patients who received neoadjuvant treatment at the Netherlands Cancer institute for Stage II–III breast cancer between 2000 and 2007. The pathological complete remission (pCR) rate was used to assess chemotherapy response. The IHC and molecular subtypes showed high concordance with the exception of the $HER2+_{IHC}$ group. 60% of the $HER2+_{IHC}$ tumors were not classified as $HER2+_{mRNA}$. The $HER2+_{IHC}$/Luminal A or B_{mRNA} group had a low response rate to a trastuzumab-chemotherapy combination with a pCR rate of 8%, while the $HER2+_{mRNA}$ group had a pCR rate of 54%. The Luminal A_{mRNA} and Luminal B_{mRNA} groups showed similar degrees of response to chemotherapy. Neither the PR status nor the endocrine responsiveness index subdivided the $ER+_{IHC}$ tumors accurately into Luminal A_{mRNA} and Luminal B_{mRNA} groups. Molecular subtyping suggests the existence of a $HER2+_{IHC}$/$Luminal_{mRNA}$ group that responds poorly to trastuzumab-based chemotherapy. For $Luminal_{IHC}$ and $triple\ negative_{IHC}$ tumors, further subdivision into molecular subgroups does not offer a clear advantage in treatment selection.

INTRODUCTION

Breast cancer is a highly heterogeneous disease and the need for individualized therapy is widely accepted. In addition to clinical parameters such as tumor size and grade, lymph node involvement and patient demographics, several molecular markers are employed in routine patient care [1–3]. The most important ones include the estrogen receptor (ER), the progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2). ER-positive tumors are thought to have characteristics of the luminal cell type and are frequently responsive to endocrine treatment (such as tamoxifen or aromatase inhibitors) [4, 5]. ER-negative tumors are considered to be more similar to the basal cell type and do not respond to endocrine treatment. Tumors with a HER2 gene amplification may respond to targeted therapy, such as trastuzumab or lapatinib [6–8]. The PR is a prognostic marker, but the Oxford overview of adjuvant therapy does not support its ability to predict resistance to chemotherapy (CT). It is sometimes stated that $ER+_{IHC}/PR+_{IHC}$ tumors coincide with the Luminal A_{mRNA} breast cancer subtype [9].

The most standardized way of assessing the status of these biomarkers is immunohistochemistry (IHC). Using antibodies with specificity for each marker, the number of positively staining cells can be estimated by the pathologist from a section of the tumor. Although widely accepted and available, the technique is not perfect. The determination of HER2 protein expression status based on IHC is known to have a false-positive rate around 10%, even in experienced laboratories [10, 11]. Many institutions also perform fluorescent or chromogenic in situ hybridization (FISH or CISH) to confirm HER2 gene amplification or to establish its presence or absence when IHC results are ambiguous. Since the choice of treatment critically depends on the HER2 gene amplification status, highly reliable analyses are essential [12]. Subtyping of breast cancer by IHC assays for ER, PR and IHC and in situ hybridization for HER2, yields three broad groups: $Luminal_{IHC}$, when ER is positive and HER2 is not amplified; $HER2+_{IHC}$ tumors, which may be ER+ or ER-; and triple negative tumors (TN_{IHC}) when ER, PR and HER2 are all negative [13, 14].

More recently, an mRNA expression-based subtyping of breast cancer, introduced by Perou et al. [15] has gained wide acceptance. These investigators identified an intrinsic gene set that distinguished five different molecular subtypes: Luminal $A_{mRNA,}$ Luminal B_{mRNA}, $HER2+_{mRNA}$, $Basal_{mRNA}$ and $Normal\text{-}like_{mRNA}$ [15–17]. Several studies have shown that the Luminal A_{mRNA} subtype is associated with a favorable prognosis, while the $Basal_{mRNA}$ subtype is prognostically unfavorable [16–18]. This raises the question of how well the two subtyping systems match and whether the molecular subtyping adds predictive power to the IHC subtyping in the neoadjuvant setting. To our knowledge, no such formal analysis has been performed. In this paper, we present the results of a comparative analysis on 195 patients.

PATIENTS AND METHODS

Patients

Biopsies of primary breast tumors were collected from 195 women who received neoadjuvant treatment at the Netherlands Cancer Institute between 2000 and 2007. These patients took part in one of two ongoing clinical trials or received standard treatment. All patients eligible for preoperative chemotherapy were diagnosed with invasive breast cancer and either a tumor diameter of at least 3 cm, lymph node involvement or both. Both trials were approved by the ethical committee and informed consent was obtained from all patients. Biopsies were taken using a core needle under ultrasound guidance. After collection, specimens were snap-frozen in liquid nitrogen and stored at -70°C.

Neoadjuvant chemotherapy for $Luminal_{IHC}$ and TN_{IHC} tumors consisted of either dose-dense AC (doxorubicin and cyclophosphamide, standard arm) or docetaxel and capecitabine (experimental arm) for three courses. After evaluation, by comparing a repeat contrast-enhanced MRI to a prechemotherapy MRI, patients with favorably responding tumors continued their initial chemotherapy and patients with minimal response or stable disease were switched to the alternative chemotherapy regimen [19]. Most tumors harboring HER2 gene amplifications were treated with trastuzumab and weekly paclitaxel and carboplatin (37 of 43 tumors). The other six patients with HER2+ tumors, who started treatment before 2006, began treatment with dose-dense AC. Details of the studies will be published separately. For four patients, no response data were available and as a result the therapy response analysis was limited to 191 patients. An overview of patient and tumor characteristics is given in Table 1.

Response evaluation

The response to treatment at the time of surgery was taken as an end point. Both pathology and MRI findings were used for response evaluation. We included both the response of the primary tumor and the nodal status after treatment in our definition of pathological response.

Only patients with a complete absence of invasive tumor cells (irrespective of carcinoma in situ) in the surgical specimen of the breast (i.e., pCR of the primary tumor) and of the lymph nodes were considered to have a pCR. It has been shown that pCR correlates with outcome and that patients achieving a pCR by this definition have a very good prognosis [20–25]. The response of the primary tumor was categorized in additional categories as described in the following paragraphs.

When only a small number of scattered tumor cells were present at pathology examination, the response was classified as a 'near pCR' (npCR). Patients with primary tumor shrinkage of more than 50% but with residual tumor were considered partial responders (PR). And at last, patients with tumor shrinkage of less than 50% as evaluated by MRI and pathological assessment were considered to be non-responders (NR). The MRIs were performed and interpreted as reported previously [19].

Table 1. Patient and tumor characteristics

Characteristic	Number (%)
Samples	195
Samples included in analysis	191
Age (years)	
Mean	46
Standard deviation	9
ER	
Positive	127 (66)
Negative	64 (34)
Node	
Positive	136 (71)
Negative	51 (27)
Not evaluated	4 (2)
HER2 gene amplification	
Positive	38 (20)
False positive	5 (3)
Negative	148 (77)
Tumor size (cm)	
<2	12 (6)
>2	179 (94)
Grade	
Low	3 (1)
Medium	61 (32)
High	57 (30)
Unknown[a]	70 (37)
IHC subtype	
Triple negative	47 (25)
HER2+	43 (22)
Luminal	101 (53)
Molecular subtype	
Basal	52 (27)
HER2+	19 (10)
Luminal A	83 (43)
Luminal B	28 (15)
Normal-like	9 (5)
Initial chemotherapy[b]	
AC	132 (69)

Table 1. *continued*

Characteristic	Number (%)
CD	24 (13)
PTC	33 (17)
Other	2 (1)

[a] For a number of biopsies the grade could not be determined
[b] AC: doxorubicin and cyclophosphamide; CD: capecitabine and docetaxel; PTC: paclitaxel, trastuzumab and carboplatin; Other: flu-orouracil, epirubicin and cyclophosphamide (FEC) or doxorubicin and docetaxel (AD)

Immunohistochemistry

Paraffin-embedded sections were immunohistochemically assessed as described previously with the following exceptions [26]. ER and PR positivity was defined as at least 10% of cells staining positive for ER or PR, respectively. The IHC staining for HER2 was scored according to standard criteria as 0, 1+, 2+ or 3+. Scores of 0 and 1+ were considered negative and 3+ was considered positive. When a score of 2+ was found, additional CISH testing was done to establish HER2 gene amplification status. CISH testing was also done when the IHC score was 3+ but no high HER2 expression was encountered in the mRNA expression microarray analysis. Tumors with at least five HER2 copies per nucleus, as detected by CISH, were considered HER2+. The tumor grade was assessed using the Elston and Ellis method [27].

Molecular subtyping

mRNA isolation and extraction from the frozen material were performed as described previously [26]. A 5-µm section halfway through the biopsy was stained for hema-toxylin and eosin and analyzed by a pathologist for tumor percentage. Only samples that contained at least 50% tumor cells were subsequently analyzed on a microarray. The microarray analysis was performed as described previously, except no filtering of genes was done [26]. Briefly, all samples were hybridized in dye-swap to in-house printed 35 k Operon microarrays using a reference pool of 100 invasive breast carcinomas. Background-corrected intensities were used to calculate $\log_2$ transformed ratios and the ratios were normalized using a lowess fit per subarray.

The subtype single sample predictor developed by Hu et al. [18.] was used to assign a molecular subtype to the samples based on their expression profiles across the intrinsic gene set. Briefly, we mapped the intrinsic genes to the Operon platform (Supplemental data file 1), when a single gene was represented by multiple probes the average of the corresponding probes was used. Subsequently, for all samples the Spearman correlation of a sample to the cen-troid of each corresponding molecular subtype was calculated. Each sample was then assigned to the subtype with the highest correlation coefficient.

Endocrine responsiveness

The endocrine responsiveness index (ERI) was defined as was described by Colleoni et al. [28]. Tumors were classified as highly endocrine responsive when ER and PR were positive in at least 50% of the cells, as incompletely endocrine responsive when either ER or PR was positive in less than 50% of the cells and as endocrine non-responsive when both ER and PR were negative in all cells.

Statistical tests

Concordance between IHC and molecular subtyping was assessed by the percentage of concordance and by the kappa test [29]. The Fisher exact test was used to assess the association between the different subtype groupings and the treatment response in terms of pCR. For the univariate and multivariate analyses, logistic regression was employed. The Cochran–Armitage exact test was used to determine trend effects. The Mann–Whitney test was used to assess PTEN mRNA expression differences between groups. All data analyses were performed using the R software package.

RESULTS

Concordance of clinical and molecular subtypes

To assess the concordance of the subtypes, we (1) disregarded the Normal-like$_{mRNA}$ group; (2) merged the Luminal A$_{mRNA}$ and Luminal B$_{mRNA}$ groups into a single group to be compared with the Luminal$_{IHC}$ group; (3) took the TN$_{IHC}$ group as the equivalent of the Basal$_{mRNA}$ group and (4) assumed equivalence of the HER2+$_{IHC}$ and HER2+$_{mRNA}$ groups. As shown in Table 2, the IHC and molecular subtypes are highly concordant except for the HER2+ groups. With the HER2+$_{IHC}$ group included, the overall concordance is 87% and the observed unweighted kappa is 74%. Without the HER2+ groups, i.e., removing all samples that were either classified as HER2+$_{IHC}$ or as HER2+$_{mRNA}$, the overall concordance increases to 97% and an observed unweighted kappa of 97%.

Table 2. IHC and molecular subtype concordance

	Basal$_{mRNA}$	HER2+$_{mRNA}$	Luminal A$_{mRNA}$	Luminal B$_{mRNA}$	Normal-like$_{mRNA}$
TN$_{IHC}$	44	1	1	0	2
HER2+/ER+$_{IHC}$	2	6 (5)	10 (9)	5 (4)	3 (2)
HER2+/ER-$_{IHC}$	5 (4)[a]	11	0	0	1
Luminal/PR+$_{IHC}$	2	1	59	14	1
Luminal/PR-$_{IHC}$	1	0	14	10	2

[a] Between brackets the number of samples remaining after removal of the false-positive HER2+$_{IHC}$

To study the Luminal$_{IHC}$–Basal$_{mRNA}$ mismatches, the ER mRNA expression levels of these samples were estimated based on the microarray hybridizations. Two of the three mismatches had the lowest ER mRNA expression of all Luminal$_{IHC}$ tumors. One of these also had relatively low ER protein expression by IHC (only 10% of nuclei) while the other had a high number of nuclei staining positive (70%). The third mismatch had both high IHC positivity (100%) and average ER mRNA expression. The single TN$_{IHC}$–Luminal A$_{mRNA}$ mismatch had the highest ER mRNA expression of all TN$_{IHC}$ tumors.

Molecular subtype assignments of the HER2+$_{IHC}$ samples

Of the 43 HER2+$_{IHC}$ samples, 7 (16%) had low HER2 mRNA expressions in the microarray analysis. To resolve this discrepancy, five of these tumors could be retested for HER2 gene amplification by IHC and CISH, and all now tested negative. For the other two samples, no tumor tissue remained from the small pretreatment biopsies. In general, however, HER2 mRNA expression and the level of HER2 positive staining showed a reasonable correlation (Supplemental Fig. 1). The only discordances were the seven samples discussed earlier. The five identified false-positive HER2+$_{IHC}$ tumors were excluded from all further analysis.

The remainder of the HER2+$_{IHC}$ samples had a moderate to high HER2 mRNA expression (36 of 43) and were scored positive based on IHC. These tumors most likely do have a HER2 gene amplification but were not all classified as HER2+$_{mRNA.}$ The molecular subtyping distributes the HER2+$_{IHC}$ tumors across all molecular subtypes. A significant proportion is classified as Luminal A$_{mRNA}$ or as Luminal B$_{mRNA.}$ Since these subtypes are largely characterized by their hormone receptor status, we next subdivided the IHC subtypes according to their hormone receptors (Table 2). All nine HER2+$_{IHC}$/Luminal A$_{mRNA}$ and all four HER2+$_{IHC}$/Luminal B$_{mRNA}$ had a positive ER by IHC. Two HER2+$_{IHC/}$ER+$_{IHC}$ tumors were classified as Basal$_{mRNA,}$ five as HER2+$_{mRNA}$ and two as Normal-like$_{mRNA}$.

Evaluation of the IHC analogs of the Luminal A$_{mRNA}$ and Luminal B$_{mRNA}$ subtypes

Since the IHC subtyping only allows for a subdivision into a Luminal$_{IHC}$, a HER2+$_{IHC}$ and a TN$_{IHC}$ group, it has been reported that PR status can be used to further subdivide the Luminal$_{IHC}$ group into a surrogate Luminal A$_{IHC}$ and Luminal B$_{IHC}$ group [9]. The reported method was employed to assign the ER+/PR+/HER2-$_{IHC}$ tumors to the Luminal A$_{IHC}$ group and the ER+/PR-/HER2-$_{IHC}$ tumors to the Luminal B$_{IHC}$ group (see Table 2). Although there appears to be some association between the IHC groups (Luminal A$_{IHC}$ and Luminal B$_{IHC)}$ and the molecular subtypes (Luminal A$_{mRNA}$ and Luminal B$_{mRNA),}$ this association is not statistically significant (Fisher exact test; P = 0.054) and the concordance is only 71% (unweighted kappa 22%). Another possibility could be that 'highly endocrine responsive' tumors classify as Luminal A$_{mRNA}$ and 'incompletely endocrine responsive' tumors as Lumi-nal B$_{mRNA}$ [28]. This is, however, not the case (Table 3, Fischer Exact test: P = 0.51; concordance 46%).

HER2+$_{IHC}$ chemotherapy response by molecular subtype

The full table of response of both breast tumor and axillary lymph nodes by IHC and by molecular subtypes can be found in Supplemental Table I. For the study of response rates, the analysis was limited to those HER2+$_{IHC}$ tumors that were treated with trastuzumab and chemotherapy. As can be seen from Table 4, the HER2+$_{IHC}$ tumors classified as either Luminal A$_{mRNA}$ or Luminal B$_{mRNA}$ have a much lower pCR rate than the non-Luminal group (P = 0.009, Fisher exact test; odds ratio: 14.7 [95% confidence interval: 1.59–135.33]).

Luminal A$_{mRNA}$ and Luminal B$_{mRNA}$ response rates

Molecular subtyping allows the separation of Luminal tumors into the Luminal A$_{mRNA}$ and Luminal B$_{mRNA}$ subgroups. One pCR was found in each subgroup (Table 5) resulting in a slightly (and not significantly) higher pCR rate in the Luminal B$_{mRNA}$ subgroup (P = 0.44, Fisher exact test).

Response rates of the primary tumor

The effect of treatment on the breast tumor alone is shown in Table 6, 7. In this overview, the Luminal groups again show a worse response than the non-Luminal groups, although more than half of the Luminal tumors are being classified as partial responders.

Table 3. Endocrine responsiveness index concordance with molecular subtypes

	Basal$_{mRNA}$	HER2+$_{mRNA}$	Luminal A$_{mRNA}$	Luminal B$_{mRNA}$	Normal-like$_{mRNA}$
ERI-	48	12	1	0	3
ERI+	2	4	39	16	2
ERI++	3	2	43	12	3

Abbreviations: ERI-, endocrine non-responsive; ERI+, incompletely endocrine responsive; ERI++, highly endocrine responsive

Table 4. Response of HER2+$_{IHC}$ tumors to trastuzumab-chemo- therapy by molecular subtype

No pCR	pCR	pCR fraction	CI	
Basal$_{mRNA}$	2	4	0.67	0.30–0.90
HER2+$_{mRNA}$	6	7	0.54	0.29–0.77
Normal-like$_{mRNA}$	1	1	0.50	0.09–0.91
Luminal A$_{mRNA}$	8	1	0.11	0.02–0.44
Luminal B$_{mRNA}$	3	0	0	0.00–0.56

Table 5. CT response rates for Luminal $_{AmRNA}$ and Luminal B_{mRNA} subtypes

	No pCR	pCR	pCR fraction	CI
Luminal A_{mRNA}	81	1	0.01	0.00–0.07
Luminal B_{mRNA}	26	1	0.04	0.01–0.18

Table 6. Response of primary tumor by IHC subtype

	NR	PR	Near-pCR breast	pCR breast
TN_{IHC}	11	17	1	18
$HER2+_{IHC}$	9	6	5	18
$Luminal_{IHC}$	50	41	5	5

Table 7. Response of primary tumor by molecular subtype

	NR	PR	Near-pCR breast	pCR breast
$Basal_{mRNA}$	12	15	1	23
$HER2+_{mRNA}$	3	6	1	8
Luminal A_{mRNA}	41	30	5	6
Luminal B_{mRNA}	10	11	4	2
$Normal\text{-}like_{mRNA}$	4	2	0	2

DISCUSSION

The relevance of molecular subtyping for breast cancer has achieved widespread acceptance. The designations 'Luminal' and 'Basal' have become part of the standard clinical terminology, although its use is often not based on microarray analysis, but rather on the routinely available tests for the estrogen- and progesterone receptors and for amplification of the HER2 gene. The four-way subtyping in ER+/HER2- tumors, triple negatives, HER2+/ER+ and HER2+/ER- tumors must be done when adjuvant or neoadjuvant treatment is considered, since it is indispensable for the selection of drug therapy. Endocrine treatments are only effective in the ER+ tumors, trastuzumab-based treatments only in the HER2+ tumors, while chemotherapy may be beneficial in all groups, particularly in the ER- tumors.

Both the clinical and the molecular subtypes have been associated with prognosis and with sensitivity to chemotherapy. For instance, it has been shown that the Luminal A_{mRNA} group has a favorable prognosis compared to the other molecular subtypes and that the $Basal_{mRNA}$ group has the worst prognosis [15, 18]. The same is true for the $ER+_{IHC}$/$HER2-_{IHC}$ and the $triple\ negative_{IHC}$ subgroups. In

addition, the $Basal_{mRNA}$ (or $triple\ negative_{IHC}$) and HER2+ molecular and IHC groups have been shown to be relatively sensitive to CT while the Luminal molecular and IHC groups are less so [30, 31]. A possible confounder in the response evaluation of our study could be the different regimens of chemotherapy that patients received. However, the different regimens are not restricted to or overrepresented in specific subtypes (with the exception of the trastuzumab-based treatment regimen) and since other studies that used the same regimen across all subtypes reported similar results, we consider the overall conclusions to be valid [30, 32]. For the day-to-day management of breast cancer with preoperative chemotherapy, the questions arises whether the additional effort and expense of true molecular subtyping is justified by an improved accuracy of response prediction.

We examined 195 tumor biopsies from breast cancer patients who were scheduled for neoadjuvant chemotherapy, and we classified the tumors according to routine clinical tests for ER and PR protein expression as well as HER2 gene amplification. Using mRNA expression microarrays, we also classified the tumors according to the molecular subtypes that have been derived from unsupervised, hierarchical clustering of primary human breast cancers. The comparison of the two subtyping systems suggests that molecular sub-typing will probably not have a major impact on treatment selection for preoperative chemotherapy for most patients with breast cancer. The Luminal and Basal molecular subtypes largely coincide with the clinical subtypes $Luminal_{IHC}$ ($ER+_{IHC}$/$HER2-_{IHC}$) and $triple\ negative_{IHC}$. The use of a molecular classification system does not appear to offer a better prediction of neoadjuvant therapy response than a simpler routine IHC/FISH based method. The further subdivision into Luminal A_{mRNA} and Luminal B_{mRNA} groups is not mirrored by the immunohistochemistry for the Progesterone receptor, nor by the differentiation between 'highly endocrine responsive' and 'incompletely endocrine responsive' tumors. In contrast to what was reported by others [33], we did not observe a significantly better response to CT in Luminal B_{mRNA} tumors in comparison to Luminal A_{mRNA} tumors. It should be noted that the sample size could obscure small, but real, differences in response rates. However, the clinical relevance of these small differences is arguable. None of the three approaches to further subdivide the $ER+_{IHC}$/$HER2-_{IHC}$ group appears to result in better predictors of chemotherapy response, despite the fact that the prognostic power of each of these has been well documented [34–39]. Although the Luminal tumors in general do not reach a pCR, a significant proportion (53%) of these achieve a reduction in primary (breast) tumor volume of at least 50%. Treatment of these tumors with chemotherapy can allow breast-conserving surgery to take place [34–39] and as such can be an effective treatment option for this group. The Normal-like subgroup is so small that no conclusions can be drawn at this moment.

Quite a different situation, however, exists in the group of tumors that harbor a HER2 gene amplification. The concordance between the $HER2+_{IHC}$ and $HER2+_{mRNA}$ subtypes is low. A small part of this lack of concordance can be explained by false-

positive HER2 IHC staining. This is not unexpected, as several studies have investigated the reproducibility of immunohistochemistry for HER2 protein expression, and poor results with false-positive rates around 10–15% have been reported [10, 11].

The remaining discrepancies are the result of intrinsic differences between the two subtyping methods. Many HER2-amplified tumors are classified as Luminal tumors in the molecular classification (34%). All $HER2+_{IHC}$ now routinely receive trastuzumab as part of the (neo)adjuvant regimen and this has proven to be very effective. In our hands, the $HER2+_{IHC/}ER-_{IHC}$ tumors are particularly sensitive to the trastuzumab/paclitaxel/carboplatin (TPC) regimen and achieve a pCR rate of 64% (Supplemental Table II). The response rate of the molecular HER2 subtype is lower (54%, Table 4) and does not improve on the clinical response prediction. Interestingly, however, the HER2-amplified tumors that are classified as Luminal by mRNA expression, have a very low pCR rate (8%, Table 4), which is lower than that of the clinically identifiable HER2+/ER+ group (21%, Supplemental Table II). In univariate analyses, only the Luminal molecular subtype and ER-status were found to be significant predictors of response (variables tested included: grade (>2), age (>48), tumor size (>T2) and lymph node involvement). In a multivariate analysis (logistic regression), the model including ER-status and Luminal molecular subtype was better in predicting response than the model with ER-status alone, but not significantly so ($P = 0.08$; Supplemental Table III). To perform a conclusive multivariate analysis, more samples will be needed.

Thus, intrinsic resistance of HER2-amplified $Lumi\text{-}nal_{mRNA}$ tumors to trastuzumab-based chemotherapy regimens may exist. Reported mechanisms of resistance to trastuzumab include altered receptor–antibody interaction, signaling by HER receptor family members, IGF1R signaling, modulation of $P27^{KIP1}$ and loss of PTEN and/or PI3K pathway activation [40, 41]. We observed that the PTEN mRNA expression in the $HER2+_{IHC/}Luminal_{mRNA}$ group tended to be higher than that in the $HER2+_{IHC}$/non-$Lumi\text{-}nal_{mRNA}$ group ($P = 0.06$, Mann–Whitney test), suggesting that PTEN inactivation has no role in this context. The number of tumors in our series is small and a recent, larger study reported more similar response rates for $HER2+_{IHC}$/$ER+_{IHC}$ and $HER2+_{IHC/}ER-_{IHC}$ patients to trastuzumab-based treatment (47 vs. 61%, respectively) than what we have found (21 vs. 64%) [42]. Although a different treatment regimen was used in that study (trastuzumab with paclitaxel and FEC) and they did not include the molecular classification in their analysis, confirmation of our finding from independent series is required. If confirmed, this finding could lead to an mRNA expression-based test on pretreatment biopsies predictive for tumor unresponsiveness to trastuzumab-based treatment. The efficacy of newer drugs that block the HER2 receptor by other mechanisms than trastuzumab, such as lapatinib, should be explored with priority in these relatively insensitive subgroups.

We conclude that the time has not yet come for the routine use of molecular subtyping in the neoadjuvant treatment setting of breast cancer. In our series of 195 patients, standard subtyping based on ER and PR status and HER2 gene

amplification performed as well and remains essential for treatment selection. In the HER2+$_{IHC}$ subgroup, mRNA expression analysis identified false-positive HER2$_{IHC}$ results, but these false positives could have been avoided by an in situ hybridization test. A separate group with low responsiveness to trastuzumab-based chemotherapy may be formed by the HER2+$_{IHC/}$Luminal$_{mRNA}$ tumors.

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SUPPLEMENTAL DATA

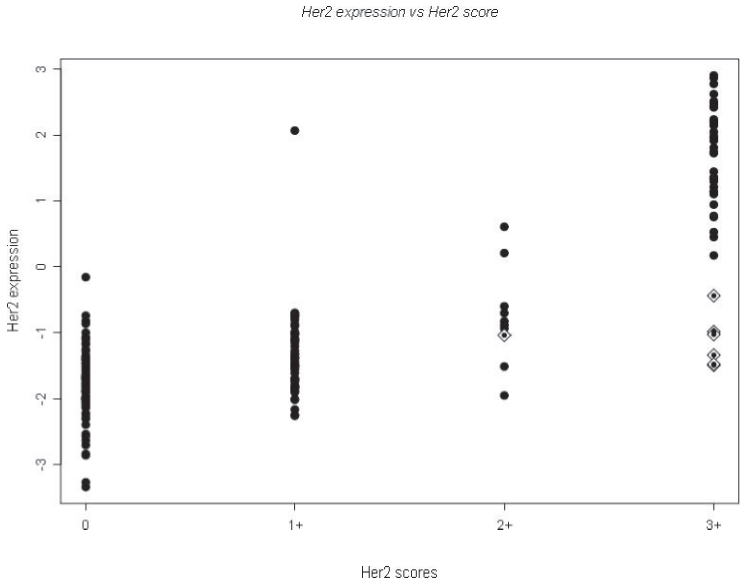

Figure 1. HER2 (log2 ratio) mRNA expression versus HER2 IHC staining. Samples that were reanalyzed (IHC and CISH) are highlighted with a diamond-shaped box.

Table I.
a. Response by IHC subtype

	no pCR	pCR	pCR fraction	CI
TN_{IHC}	32	15	0.32	0.20 – 0.46
$HER2+_{IHC}$	25	13	0.34	0.21 – 0.50
$Luminal_{IHC}$	99	2	0.02	0.01 – 0.07

b. Response by molecular subtype

	no pCR	pCR	pCR fraction	CI
$Basal_{mRNA}$	31	20	0.39	0.27 – 0.53
$HER2+_{mRNA}$	11	7	0.39	0.20 – 0.61
Luminal A_{mRNA}	81	1	0.01	0.00 – 0.07
Luminal B_{mRNA}	26	1	0.04	0.01 – 0.18
$Normal\text{-}like_{mRNA}$	7	1	0.13	0.02 – 0.47

Table II. Response by ER_{IHC} status for $HER2+_{IHC}$ patients receiving trastuzumab based treatment

	no pCR	pCR	pCR fraction	CI
$HER2+/ER+_{IHC}$	15	4	0.21	0.09 – 0.43
$HER2+/ER-_{IHC}$	5	9	0.64	0.39 – 0.84

Table III. Multivariate analysis of predictive factors for pathological CR after trastuzumab based treatment in $HER2+_{IHC}$ patients

	Model 1* Wald statistics		Model 2** Wald statistics	
Variable	Chi-square	p	Chi-square	p
ER-status		0.016		0.35
Molecular subtype***		-		0.10
Total model	5.81	0.016	6.41	0.041

* Model 1: ER-status only; 1 degree of freedom
** Model 2: ER-status and Molecular subtype; 2 degrees of freedom
*** Luminal versus non-Luminal

Molecular subtyping of breast cancer: ready to use?

Genomic analysis has increased substantially the potential for individualised treatment of patients with breast cancer. Perou and colleagues [1] took the first step in genome-wide molecular characterisation of breast carcinomas in 2000. On the basis of mRNA expression profiles, five distinct subtypes have emerged since: luminal A, luminal B, HER2-enriched, basal-like, and normal-like[2–4], a taxonomy that has become widely used in breast-cancer research. These classes have distinct prognoses and responses to chemotherapy [4], and it has been argued that gene expression is the gold standard for the identification of breast-cancer subtypes [5]. Clinical trials that stratify for molecular subtype have already been set up (eg, ClinicalTrials.gov NCT00546156, NCT00991263). With a single sample predictor (SSP), individual patients can be assigned to a molecular subtype [3].

In this issue of *The Lancet Oncology*, Weigelt and colleagues [6] compare three such SSPs and assess their concordance. This research is highly relevant because molecular subtypes in different studies are assumed to represent the same entities [5]. With various datasets these researchers show that, although survival trends for subtypes are similar across SSPs, individual assignments are not, with the exception of the basal-like class.

The finding that the basal-like subtype is the only class with a high concordance across all SSPs and datasets is very interesting, because it suggests that this subtype represents a well-defined entity that can be identified with high confidence. On the other hand, Weigelt and colleagues did not find any normal-like breast carcinomas in a series of specimens from which tumour cells were isolated by microdissection, strongly suggesting that this molecular subtype, as initially described, is due to an admixture with normal cells. In general, growth pattern relates to the proportion of tumour cells in analysed material. For example, samples from most high-grade basal-like tumours frequently show an expansive growth pattern and therefore samples will have a fairly high proportion of tumour cells, whereas the opposite is true for diffusely infiltrating, low-grade, luminal A cancers. This difference in tumour cell percentages will definitely have an effect on the robustness of SSPs. To the best of our knowledge, this has never been studied in great detail.

HER2-positive tumours are fairly sensitive to trastuzumab-based treatment. Disappointingly, the three SSPs classified only 0%, 0%, and 54% of HER2- positive—as assessed by immunohistochemistry—grade III tumours from the researchers' own dataset as HER2- enriched. This finding poses an important challenge to clinical use of molecular subtyping. However, some evidence [7] suggests that this technique could identify poor responders to neoadjuvant trastuzumab-based treatment in the HER2-amplified group, indicating a potential complementary role of molecular subtyping.

For individual patients, the molecular subtype will be dependent on the SSP used, which is clearly not a desirable feature when stratifying participants for a

clinical trial. Which, if any, is the true classification of a tumour? Weigelt and colleagues show that prognostic performance is similar between SSPs and, in that sense, no predictor seems to be superior. The clinical relevance of molecular subtyping inevitably lies in therapeutic outcomes, either sparing patients with very good prognosis the side-effects of treatment or actually selecting a subgroup who are likely to respond to a (targeted) therapy. mRNA-based molecular subtyping was mainly aimed at grouping tumours with similar gene-expression profiles together. Although these groups show similar survival trends across SSPs, their therapeutic relevance still needs to be proven.

In conclusion, molecular subtyping has not yet matured sufficiently for stable stratification of luminal and HER2-enriched breast carcinomas. Furthermore, it has limited clinical relevance for subtyping of basal-like breast tumours because of the large overlap between triple-negative and basal-like cancers [7,8]. For now, other means—ie, immunohistochemistry for HER2 and oestrogen receptor status, and array comparative genomic hybridisation to test for *BRCA* gene status and homologous recombination deficiency—are of more predictive value in clinical decision-making for neoadjuvant and adjuvant systemic therapy. Therefore, molecular subtyping should not be used instead of morphology and immunohistochemistry but rather in addition to these classic approaches, to increase clinical relevance and robustness.

The authors declared no conflicts of interest.

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