Correlation of HER2 expression by IHC, DNA microarray, and FISH in breast cancer

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Introduction

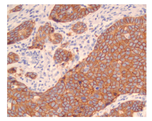
HER2 overexpression occurs in approximately 15-20% of pts with breast cancer and is associated with aggressive disease and decreased survival. HER2 status is predictive of response to trastuzumab and lapatinib. Given the importance of HER2 positive disease, accurate evaluation of HER2 status is essential. The aim of this study is to provide insights into the relationship between HER2 expression by immunohistochemistry, DNA microarray and FISH in a large cohort of 1,032 breast cancers. HER2 protein expression were determined using antibody clones (4B5) and interpreted per ASCO/CAP scoring criteria. Samples scored as equivocal (>=2+;> 10% to <+3+; <=30%) were required to undergo further assessment with FISH HER2 testing (Pathvysion). All samples were tested for HER2 by DNA microarray provided there was sufficient quantity of RNA in all tumor samples.

Investigation of two-way comparisons using qualitative (categorial) outcome measures (overexpressed, underexpressed, equivocal) yielded statistically significant results but demonstrated poor kappa values, showing only slight agreement between the assays (IHC/FISH K=.096, p<.001; FISH/Microarray K=.079, p<.05; IHC/Microarray K=.126, p<.001). In-depth analyses indicate that equivocal or negative protein expression trend towards non-amplification (76.9%, n=837 and 90.5% n=182, respectively, p< .001). Low RNA levels are most often associated with IHC negativity (61.2%, n=762) but are also noted in samples with HER2 overexpression (38.4%, n=478). The least structured relationship was observed between DNA Microarray and FISH. Pearson correlation coefficients, based on intensity of staining, Quick score (IHC), gene ratio (DNA Microarray) and HER2-FISH ratio indicate that both IHC Quick score and intensity of staining exhibit modest correlations (r=.529, n=979, p<.001; r=.486, n=981, p<.001, respectively). This is well evidenced in the association between HER2 protein expression and median HER2-FISH ratio, which is 5.32 (SD=2.47) in overexpressed and 1.06 (SD=.34) in underexpressed samples, even when controlling for equivocal results (Kruskal Wallis <.001, n=142). DNA Microarray showed the least concordance with the other assays (FISH r=.440, n=573, p<.001), IHC/ Quick score r=.377, n=573, p<.001; IHC/intensity r=.342, n=573, p<.001).

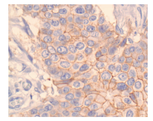
To conclude, our data suggest that - due to poor inter-assay agreement - breast cancers with HER2 IHC and HER2 FISH equivocal results cannot be resolved by DNA Microarray. Protein expression by IHC is the most robust and cost effective way to test for HER2 expression when performed per ASCO/CAP guidelines and it correlates well with gene amplification by FISH.

Materials/Methods

A large cohort of 1,032 breast cancer specimens (core needle biopsies or surgical specimens) were analyzed for HER2 by IHC and – if sufficient tissue remained - DNA microarray and/or FISH. Ventana's HER2 (4B5) antibody was utilized for immunostaining, with pathologists using cut-offs [above threshold, below threshold (equivocal), and negative] based on ASCO/CAP guidelines for breast cancer:



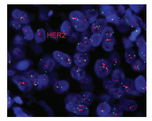
HER2 Above Threshold
(3+ and >=30%)



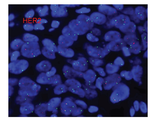
HER2 Below Threshold
[(2+ and >10%)
OR (<3+ and <30%)]

DNA microarray (DASL process, Illumina) was performed on the ERBB2 (HER2) gene whenever possible. Results of gene overexpression, underexpression, and "no change" (no difference in expression) were based on a tissue-specific normal control. Results of "not performed" were secondary to insufficient RNA for microarray analysis, and results considered "not informative" indicated that data obtained from either the patient sample or the control sample were not of sufficiently high quality to confidently evaluate gene expression.

HER2 by FISH (Pathvysion HER2 DNA Probe Kit, Abbott Laboratories) was performed when IHC results were considered above threshold or below threshold (equivocal), in concordance with ASCO/CAP guidelines for amplification:



FISH amplification

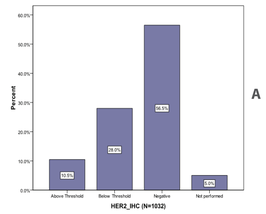


No FISH amplification

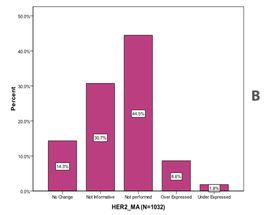
All statistical analyses were performed using the IBM SPSS Statistics 17 software. Inter-assay agreement (Inter-Category Variation) was assessed using Kappa statistics. All assays were trichotomized as follows: above, below, negative threshold (IHC), amplified, equivocal, not amplified (FISH) and over expressed, no change, under expressed (Microarray). Kappa values < 0.0 were considered showing poor, 0.0 – 0.20 slight, 0.21-0.40 fair, 0.41 – 0.60 moderate, 0.61 – 0.80 substantial, and 0.81 – 1.00 almost perfect correspondence. Pearson correlation coefficients were utilized for quantitative outcome measures based on IHC intensity or staining, IHC Quick* score, DNA microarray gene ratio, and FISH ratio. Pearson's correlation reflects the degree of linear relationship between two variables, with a correlation of +1 denoting a perfect positive linear relationship between variables.

Results

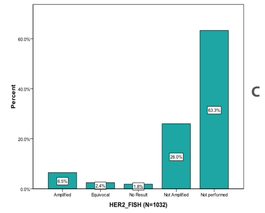
Distribution of HER2 Results by Assay



A



B



C

Figure 1A - C: The three graphs pictured on the left illustrate qualitative distribution results. In Figure 1A, HER2 by IHC exhibits a high informative rate, with only 5.1% of IHCs showing no result. Figure 1B, by contrast, indicates no change in gene expression in 89.5% of specimens tested by DNA microarray, providing no direction in patient management in the majority of tumors. In Figure 1C, the high level of HER2 by FISH results that were "not performed" is secondary to ASCO/CAP guidelines, which mandate HER2 by IHC be "above threshold" or "below threshold" to justify HER2 by FISH testing. When only "reflexed" HER-2 results are taken into consideration (n= 1449; graph not shown), 217 (15.0%) specimens were amplified, 122 (8.4%) were equivocal and 1110 (76.6%) were not amplified.

Inter-assay agreement

Distribution of (HER2) IHC and FISH			HER2 FISH: Amplified	Equivocal	Not Amplified	Total
HER2 IHC	Above Threshold	n	20	0	2	22
		%	90.90%	0.00%	9.10%	100.00%
	Below Threshold	n	52	21	222	295
		%	17.60%	7.10%	75.30%	100.00%
	Negative	n	1	3	51	55
		%	1.80%	5.50%	92.70%	100.00%
Total		n	73	24	275	372
		%	19.60%	6.50%	73.90%	100.00%

A

Distribution of (HER2) IHC and Microarray			HER2 Microarray: Over-expressed	No Change	Under-expressed	Total
HER2 IHC	Above Threshold	n	46	6	5	57
		%	80.70%	10.50%	8.80%	100.00%
	Below Threshold	n	54	56	7	117
		%	46.20%	47.90%	6.00%	100.00%
	Negative	n	81	153	63	297
		%	27.30%	51.50%	21.20%	100.00%
Total		n	181	215	75	471
		%	38.40%	45.60%	15.90%	100.00%

B

Distribution of (HER2) FISH and Microarray			HER2 Microarray: Over-expressed	No change	Under-expressed	Total
HER2 FISH	Amplified	n	17	7	0	24
		%	70.80%	29.20%	0.00%	100.00%
	Equivocal	n	3	3	0	6
		%	50.00%	50.00%	0.00%	100.00%
	Not Amplified	n	22	44	1	67
		%	32.80%	65.70%	1.50%	100.00%
Total		n	42	54	1	97
		%	43.30%	55.70%	1.00%	100.00%

C

Table 1A - C: The three tables on the left show the actual and relative distribution of HER2 outcomes by IHC, DNA microarray, and FISH. Two way comparisons (Kappa, K) on trichotomized variables indicated only poor to slight agreement between assays. The weakest correspondence was found between FISH/microarray (K=0.079, n=77), followed by IHC/FISH (K=0.096, p<0.001, n=341), whereas IHC/Microarray (K=0.126, p<0.001, n=252) appeared to show a slightly better interrelationship. However, nominal variables allow for only qualitative classification. All comparisons were therefore also subjected to quantitative analyses by Pearson Correlation.

Correlation of HER2 protein expression and gene amplification			Gene amplification by FISH: 2-2.2	2.3-2.7	>=2.8	Total
HER2_IHC	2+ (1%-29%)	N	1	2	20	23
		%	4.3%	8.7%	87.0%	100.0%
	2+ (11%-30%)	N	1	0	24	25
		%	4.0%	.0%	96.0%	100.0%
	3+ (31%-50%)	N	0	0	1	1
		%	.0%	.0%	100.0%	100.0%
	3+ (51%+70%)	N	0	0	0	0
		%	100.0%	.0%	.0%	100.0%
	3+ (>70%)	N	15	0	0	15
		%	100.0%	.0%	.0%	100.0%
Total		N	18	2	45	65
		%	27.7%	3.1%	69.2%	100.0%

Table 2: Correlation of HER2 protein expression and gene amplification. Results are discussed below in Table 3.

Results

Two-way comparison of quantitative values

IHC	ratio (Microarray)				ratio (FISH)			
HER2 Intensity	N	Median	Minimum	Maximum	N	Median	Minimum	Maximum
0	129	0.98	0.09	6.00	7	1.00	0.88	1.11
1	189	1.15	0.21	5.22	27	1.06	0.22	2.40
2	186	1.27	0.24	11.61	90	1.10	0.00	8.81
3	65	2.98	0.76	38.67	19	5.32	0.00	11.66
Total	569	1.22	0.09	38.67	143	1.11	0.00	11.66
Quick HER2	**N**	**Median**	**Minimum**	**Maximum**	**N**	**Median**	**Minimum**	**Maximum**
0	3	1.10	0.98	1.26	3	1.07	0.95	1.07
1	1	1.12	1.12	1.12	0			
2	10	1.35	0.73	2.57	5	0.94	0.65	2.40
3	75	1.27	0.27	3.36	13	1.06	0.77	1.25
4	96	1.12	0.21	5.08	27	1.07	0.00	2.76
5	213	1.04	0.09	6.00	34	1.11	0.00	2.30
6	70	1.32	0.24	5.97	25	1.07	0.00	2.82
7	41	1.78	0.81	11.61	20	1.33	0.72	8.81
8	58	3.73	0.76	38.67	15	5.57	2.56	11.66
Total	567	1.22	0.09	38.67	142	1.11	0.00	11.66

Table 3: The table above provides the median values for gene ratio (Microarray and/or FISH) by IHC intensity and Quick score. While the numbers indicate a positive trend between increases in staining intensity and ratio (independent of assay), the relationship does not appear to be exclusively linear. The highest concordance is evident when high intensity and high Quick score are taken into consideration. On a limited subset – illustrated in Table 2 - results show that very high IHC intensity and staining (cases with greater that 3+, 50%) is exclusively related to FISH amplification (ratio > 2.2), which indicates improved concordance in Her2 3+ tumors. These results are in line with Shah *et al.* 2010.

Distribution between IHC intensity, IHC Quick score, DNA microarray gene ratio, and FISH ratio

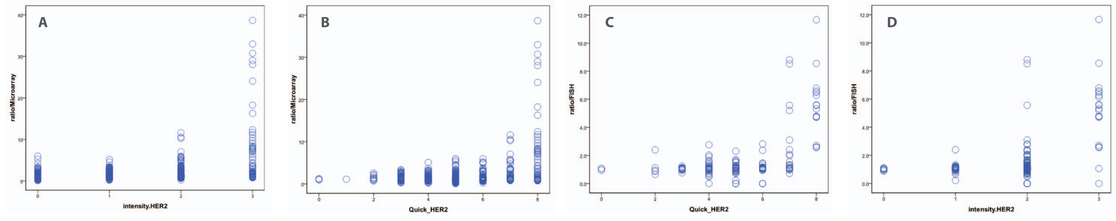


Figure 2A - D. Figures 2A and 2B provide a visual presentation of the distribution between intensity of staining, Quick score and microarray gene ratio. Graph A demonstrates that intensity of 0 and 1 are associated with similarly low gene ratios, whereas the spread becomes significantly larger for higher intensities (2+). A similar trend is observed when the Quick score is considered – again, only the highest scores (7+) show a notable increase in the gene ratio. When FISH ratios and intensity and Quick score are compared, the same trends are observed (figures 2C and 2D), with intensity of staining and FISH ratios showing a very nice separation, which is supported by guideline recommendations. In two-way comparisons, Pearson Correlation coefficients (2-tailed) - based on intensity or percent of staining, Quick score (IHC), gene ratio (DNA Microarray) and HER2-FISH ratio – indicated that HER2 staining intensity showed the highest correlation with FISH (r=.486, p<.001, n=143) and the lowest with Microarray (r=.342, p<.001, n=569). For percent staining, a similar and even more notable trend is observed (r=.513, p<.001, n=142 and r=.208, p<.001, n=567, respectively). The highest correlation was noted between IHC Quick score and FISH ratio (r=.521, p<. 001, n=142), suggesting that a composite score may be superior (for Quick score and microarray, r=.377, p<.001, n=567). Gene ratio by FISH and Microarray showed a correlation of r=.440 (p<.001, n=107). The above results consistently show that IHC and FISH – independent of scale – show the highest reciprocity.

***Quick score** = A score for the proportion of stained cells (0 = no nuclear staining, 1 = < 1% nuclear staining, 2 = 1%-10% nuclear staining, 3 = 11%-33% nuclear staining, 4 = 34%-66% nuclear staining and 5 = 67%-100% nuclear staining) and the intensity of staining (0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining) were assigned to each tumor. The score for the proportion of cells stained and the score for the intensity of staining were then added to obtain the total score, which can range from 0 to 8

Conclusions

Our data suggest the following:

- Based on poor inter-assay agreement, breast cancers with HER2 IHC and HER2 FISH "equivocal" results cannot be resolved by DNA microarray.
- Khoury *et. al.* 2011 showed that a higher concordance rate between IHC and FISH could be achieved by expanding the equivocal range to include all cases showing a 2+ intensity. However, our results show that expanding the equivocal range to include even those IHCs whose intensities were 2+ in less than 10% of cells did not support such a conclusion.
- Our results suggest that an intensity score of 3+ in 70% of cells in IHC shows the highest concordance with FISH, higher than the ASCO/CAP guidelines and consistent with Shah *et. al.* 2010.
- Protein expression – as measured by IHC – is the most robust and cost-effective way to test for HER2 expression when performed per ASCO/CAP guidelines, consistent with prior publications from various labs throughout the world such as Chibon *et. al.* 2009, Shah et. al. 2010, and Umemura *et. al.* 2008. Our protocol did not discriminate between core needle biopsies or surgical specimens, as justified by Lebeau *et. al.* 2010, which showed good results with core needle biopsy when compared with surgical specimens.
- HER2 performed by IHC correlates well with gene amplification by FISH. This is also consistent with prior publications such as Panjwani *et. al.* 2010.

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