Laboratory Testing for Her2 Status in Breast Cancer

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Overview

- Clinical relevance of Her2 status for treatment of breast cancer

- Standard approaches for determining Her2 status in breast cancer

- Current concepts and controversies in Her2 testing
Who gets breast cancer?

- Breast cancer is one of the most common malignancies to affect women

- About 1 in 8 women will be diagnosed with breast cancer at some point in her lifetime

- Most cases of breast cancer are sporadic, but a small percentage (5-10%) are related to a heritable gene mutation, most commonly *BRCA1* or *BRCA2*

- Having a first degree relative with breast cancer increases a woman’s chance of developing breast cancer

- Screening mammography is recommended for older women
  - US Preventive Services Task Force: Every 2 years starting at age 50
  - American Cancer Society, others: Every 2 years starting at age 40
How is breast cancer treated?

- Surgery: excision with or without sentinel lymph node biopsy
  - Breast conserving: lumpectomy, partial mastectomy
  - Mastectomy
- Chemotherapy: before and/or after surgery
- Radiation

- Targeted therapies
  - Hormone therapy: Tamoxifen, aromatase inhibitors
  - Her2 targeted therapy for cancers with overexpression of the gene \textit{ERBB2}, commonly called Her2 or Her2/neu

- Treatment is based on testing for ER, PR, and Her2 status, as well as cancer grade and stage.
Her2 targeted therapy

- Herceptin (trastuzumab)
- Others: pertuzumab (Perjeta), T-DM1 (Kadcyla), and lapatinib (Tykerb)
- Recent data shows that a combination of pertuzumab, trastuzumab, and docetaxel (PTD) improved progression free survival compared to patients who had only trastuzumab and docetaxel (TD)\(^1,2\)


source: [http://www.perjeta.com/hcp/moa](http://www.perjeta.com/hcp/moa)
ER, PR, and Her2

- Proteins made by some breast cancers
- ER and PR: Hormone receptors
  - ER: estrogen receptor
  - PR (PgR): progesterone receptor
  - Tested by immunohistochemistry; immunoreactivity in 1% or more cancer cells is considered positive\(^1\)
- Her2: Growth factor receptor
  - Encoded by gene \textit{ERBB2}, also known as Her2/neu, V-Erb-B2 Avian Erythroblastic Leukemia Viral Oncogene Homolog, etc.
  - Tested by immunohistochemistry and/or \textit{in situ} hybridization

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Methods for assessing Her2 status in breast cancer: Immunohistochemistry

- Antibody directed to Her2 protein, detected with a secondary antibody conjugated to a substrate (horseradish peroxidase)
- Chromogen (DAB) is used to generate stain where Her2 protein binds primary + secondary antibody

Adapted from HercepTest™ Interpretation Manual (Dako)
Methods for assessing Her2 status in breast cancer: Immunohistochemistry

- Staining intensity is correlated to the number of Her2 protein molecules per cell
- Scored according to the intensity and completeness of staining of the cell membrane, where Her2 protein resides
  - Negative (0 or 1+)
  - Equivocal (2+)
  - Positive (3+)

Her2 positive
3+ staining intensity
HercepTest (Dako)
Pros and Cons of Her2 Immunohistochemistry

• Pros
  – Inexpensive
  – Detects Her2 overexpression regardless of mechanism
  – Can visualize with brightfield microscopy under low power, allowing rapid assessment of entire tissue sample tested

• Cons
  – False negatives will be undetected due to lack of internal control
  – Subjective, semi-quantitative interpretation
Methods for assessing Her2 status in breast cancer: *In situ* hybridization

- FISH: fluorescent labeled probe
- Brightfield in situ hybridization is similar but uses non-fluorescent labeling to allow visualization by brightfield microscopy

source: http://en.wikipedia.org/wiki/Fluorescence_in_situ_hybridization
Methods for assessing Her2 status in breast cancer: *In situ* hybridization (FISH)

- FISH slide is scored by enumerating signals for the target (Her2) and the control (CEP17) (chromosome 17 centromere)
- Her2/CEP17 ratio and average Her2 signal count per cell are both used to determine Her2 status
  - Amplified
  - Non-amplified
  - Equivocal
  - Indeterminate

Her2 positive  
Her2/CEN-17 ratio ≥2  
HER2 IQFISH pharmDx™
Pros and Cons of Her2 FISH

• Pros
  – Less subjective than immunohistochemistry; an absolute quantitative score is generated
  – Her2 amplification by FISH correlates well with overexpression by IHC in breast cancer
  – Direct genetic evaluation of individual tumor cells *in situ* on a slide, allows for evaluation of cell to cell variability, sub-clonal populations

• Cons
  – More expensive than immunohistochemistry
  – More time consuming interpretation
  – Analytic difficulties related to control locus, which can also be abnormal in cancer

- Standardization of immunohistochemistry and FISH assays
- Specified tissue handling and formalin fixation times
- Mandated external proficiency testing
- Defined 3 categories of results:

<table>
<thead>
<tr>
<th>Method</th>
<th>Negative</th>
<th>Equivocal</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>No staining or weak, complete membrane staining &lt;10%</td>
<td>Weak, non-uniform staining ≥10% or Uniform intense membrane staining ≤30%</td>
<td>Uniform intense membrane staining &gt;30%</td>
</tr>
<tr>
<td>FISH</td>
<td>Single probe: &lt;4/cell</td>
<td>Single probe: 4-5.9/cell</td>
<td>Single probe: ≥6.0/cell</td>
</tr>
<tr>
<td></td>
<td>Dual probe: Ratio &lt; 1.8</td>
<td>Dual probe: Ratio 1.8-2.2</td>
<td>Dual probe: Ratio &gt;2.2</td>
</tr>
</tbody>
</table>
ASCO CAP Updated Guidelines (2013)

- Re-defined 3 categories of results:

<table>
<thead>
<tr>
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<th>Negative</th>
<th>Equivocal</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>IHC</td>
<td>No staining or faint, barely perceptible</td>
<td>Incomplete and/or weak to moderate membrane</td>
<td>Uniform intense membrane staining &gt;10%</td>
</tr>
<tr>
<td></td>
<td>staining</td>
<td>staining &gt;10%, or uniform intense</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>membrane staining ≤10%</td>
<td></td>
</tr>
<tr>
<td>FISH</td>
<td>Single probe: &lt;4/cell</td>
<td>Single probe: 4-5.9/cell</td>
<td>Single probe: ≥6.0/cell</td>
</tr>
<tr>
<td></td>
<td>Dual probe: &lt;4/cell, and ratio &lt;2.0</td>
<td>Dual probe: 4-5.9/cell, and ratio &lt;2.0</td>
<td>Dual probe: Ratio ≥2.0 or ≥6.0/cell</td>
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<td></td>
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</table>

- Created new category: Indeterminate (technical issues preventing interpretation of test)
What Changed in the 2013 Guidelines?

• Negative:
  – Immunohistochemistry: Re-defined 0 and 1+
  – FISH: *Ratio <2.0 and <4 average Her2 copies per cell* (was ratio <1.8)

• Equivocal:
  – Immunohistochemistry: ≤10% intense membrane staining (was <30%)
  – FISH: *4 to 5.9 average Her2 copies per cell* (was ratio 1.8-2.2)

• Positive:
  – Immunohistochemistry: >10% intense membrane staining (was ≥30%)
  – FISH: *Ratio ≥2.0 or ≥6 average Her2 copies per cell* (was ratio >2.2)
Additional Changes in the 2013 Guidelines

• Included guidance on new technologies
  – Brightfield in situ hybridization: guidelines same as FISH
  – DNA microarray and mRNA expression assays: insufficient evidence to support clinical use for Her2 status

• Resolved discordance between different existing methodologies
  – Single vs. dual probe FISH assays

• Minimized false negatives by lowering thresholds for equivocal and amplified/positive

• Broadened recommendations: Her2 testing on all primary and recurrent/metastatic breast cancers

• Promoted early testing of all breast cancers (diagnostic biopsy instead of excision)

• Provided route for resolving discrepancies between Her2 testing and histology

• Updated definition of genetic heterogeneity
Clinical Impact of Changed Guidelines

HER2 Amplification Status of IHC Equivocal (2+) Cases by Percent Membrane Staining

<table>
<thead>
<tr>
<th>IHC 2+</th>
<th>FISH Amplified (2013 Guidelines) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2+ Cases (All)</td>
<td>58/707 (8.2%)</td>
</tr>
<tr>
<td>2+ Cases, 10-30% Membrane Staining</td>
<td>38/507 (7.5%)</td>
</tr>
<tr>
<td>Strong</td>
<td>1/8 (12.5%)</td>
</tr>
<tr>
<td>Moderate</td>
<td>17/136 (12.5%)</td>
</tr>
<tr>
<td>Weak</td>
<td>20/363 (5.5%)</td>
</tr>
</tbody>
</table>

Poster presentation at San Antonio Breast Cancer Symposium, December 2014
Patterns of Her2 amplification

intrachromosomal ("stacked" signals)

extrachromosomal

co-amplification
Challenges in Her2 FISH Testing
1. Polysomy / Co-amplification
Chromosome 17 “polysomy” in Her2 FISH testing

- Extra copies of chromosome 17 centromere
- 3 or more copies of CEP17 (avg/cell): ~8% of cases, mostly those with 4-6 Her2 copies per cell (equivocal range)\(^1\)

1. Wolff AC et al. ASCO/CAP guideline for Her2 testing (2007)
What is “polysomy”? 

- Extra whole copies of a chromosome 
- Normal diploid state is 2 copies 
- 3 or more copies is polysomy 
- Polysomy is harder to define on FFPE sections due to signal truncation 
  - Average signal count for diploid state is < 2 in FFPE 
  - Polysomy has been defined in the medical literature as average signal counts as low as 1.86\(^1\) and ranging up to >3 
  - Most commonly adopted threshold is mean of ≥3 CEP17 signals per nucleus\(^2\)

Does chromosome 17 “polysomy” affect Her2 expression?

- Most cases **not** associated with Her2 protein or mRNA overexpression\(^1,2\)

<table>
<thead>
<tr>
<th>IHC</th>
<th>CEP17+</th>
<th>Mean HER2 Copy</th>
<th>Mean CEP17</th>
<th>Mean HER2/CEP17 Ratio</th>
<th>ISH for HER2 mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>0–1+</td>
<td>39/56 (69%)</td>
<td>4.2</td>
<td>3.1</td>
<td>1.4</td>
<td>16/16 without HER2 gene overexpression</td>
</tr>
<tr>
<td>2+</td>
<td>15/56 (27%)</td>
<td>4.6</td>
<td>3.0</td>
<td>1.5</td>
<td>9/9 without HER2 gene overexpression</td>
</tr>
<tr>
<td>3+</td>
<td>2/56 (3%)</td>
<td>4.5</td>
<td>3.1</td>
<td>1.5</td>
<td>1/1 without HER2 gene overexpression</td>
</tr>
</tbody>
</table>

- Absolute Her2 signal number per cell of 6.0 or greater is correlated with overexpression of Her2, regardless of the Her2/CEP17 ratio\(^2\)

1. Downs-Kelly et al, AJSP 2005 Sep;29(9):1221-7. (data shown above: Table 2)
Where are the extra copies of chromosome 17 centromere?

- In extra whole copies of chromosome 17 ("true polysomy")
- In extra structurally abnormal (deleted, duplicated, rearranged) DNA molecules:
  - Extra structurally abnormal copies of chr17 (centromere 17 present)
  or
  - Separate “marker” (structurally abnormal, unidentifiable) chromosomes
  - Co-amplification: discrete segments of the genome are amplified together, often in tandem on the same chromosome or on separate “marker” (structurally abnormal, unidentifiable) chromosomes
FISH with SMS/RARA probes to resolve Her2 status

Multiplex ligation-dependent probe amplification (MLPA) – chromosome 17

Microarray CGH: chromosome 17

Marchio et al, J Pathol. 219: 16-24, 2009

Green: gain
Blue: amplification
Gray: no change
(White): deletion

Graphic courtesy of Wei Shen

Blue: gain
Red: loss
Blue: gain
Red: loss

Graphic courtesy of Wei Shen & Lisa Collins
Copy number gains in the context of the cancer genome

• Entire genome may be present in 3 or more copies (on average), i.e. "polyploidy," confounding the definition of "normal" or "control" for the genome
  – Polyploidy may not be detected on microarray analysis, depending on the software tools and bioinformatic approach used for analysis

• Adult solid tumors are known to have complex genomes, characterized by gains, losses, allelic imbalances encompassing large portions of the genome

• Absolute copy number per cell can be estimated by some techniques, but not others
  – FISH, flow cytometry, cytogenetics: individual cell analysis

• Reference/ "control" region(s) may also be abnormal
(The Search for a Perfect Control)

- CEP17 is co-amplified in a fraction of cases
- Another gene region on chromosome 17 may be used as a control

  *But*.....

- No region of the genome is immune to copy number changes in cancer

  *And*...

- Chromosome 17 is especially prone to copy number changes in breast cancer
Resolution of Equivocal Her2 FISH

- ASCO-CAP 2013 Guidelines recommend using an alternate control probe for a gene on chromosome 17

- What if the alternate control probe is also abnormal (deleted or amplified)?
  - No guidelines on interpretation or further reflex testing
## Cut-off values for alternate control probe

<table>
<thead>
<tr>
<th>Study</th>
<th>Probe(s) used</th>
<th>Cutoff: Deleted</th>
<th>Cutoff: Amplified</th>
</tr>
</thead>
<tbody>
<tr>
<td>Troxell (2006)</td>
<td>SMS (RAI1), RARA</td>
<td>none</td>
<td>Not specified</td>
</tr>
<tr>
<td>Tse (2011)</td>
<td>SMS (RAI1), RARA, TP53</td>
<td>none, highest of 3 probes &lt;2.6 used as new control to calculate Her2 ratio</td>
<td>≥2.6</td>
</tr>
<tr>
<td>Mansfield (2013)</td>
<td>D17S122 (PMP22)</td>
<td>none</td>
<td>Not specified*</td>
</tr>
</tbody>
</table>

* CEP17 ≥6.0 was defined as co-amplification of chromosome 17 centromere
Alternate control probes used to resolve Her2 double equivocals

Blue: gain
Red: loss

17p

17q

centromere (CEP17)

TP53 (p53)
RAI1 (SMS)
D17S122 (PMP22)
RARA (RARA)
ERBB2 (Her2)
Reflex FISH Testing for Double Equivocals

12/773 (1.6%) in TCGA study copy number alterations at RAI1
Challenges in Her2 FISH Testing

2. Genetic Heterogeneity

Fig. 1A from Starczynski et al. HER2 gene amplification in breast cancer: a rogues' gallery of challenging diagnostic cases: UKNEQAS interpretation guidelines and research recommendations. *Am J Clin Pathol.* 137, 595-605, 2012.
Genetic Heterogeneity

- A subpopulation of tumor cells shows amplification, while the rest of the tumor is non-amplified

- 2009 guideline: More than 5% but less than 50% infiltrating tumor cells with a ratio higher than 2.2
  - Must report % amplified, pattern (scattered or discrete population) and whether cells are histologically distinctive
  - Problems with spurious “amplified” cells defined only by ratio of individual cells (e.g. 1 green and 3 red signals)

- 2013 update: More than 10% infiltrating tumor cells with increased Her2 signals/cell
  - Only reported if there is a discrete subpopulation of amplified cells, and score the amplified and non-amplified cell populations separately

Summary

- Immunohistochemistry and in situ hybridization (ISH, FISH) are the recommended methods for determining Her2 status for treatment with Her2-targeted therapy.

- Neither method is 100% sensitive or specific.

- Updated ASCO-CAP (2013) guidelines have resulted in an increased proportion of patients being eligible for Her2-targeted therapy.

- Her2-positive cases are not a homogeneous group.
  - Borderline positive cases may not be as responsive to Her2-targeted therapy.

- Challenges in Her2 laboratory testing include polysomy / co-amplification, and genetic heterogeneity.