Standardisation of HER2 analysis in carcinoma: Breast Cancer
Lessons learned, lessons forgotten:

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HER2 testing in breast cancer
1. The rationale for HER2 testing
2. Which test is best?
   - FISH or IHC?
3. Chromosome 17
4. Can I use CISH please?
5. Rogues!

What is HER2?
- Epidermal Growth factor receptor homologue
- Type 1 receptor tyrosine kinase (RTK) family member:
  - Extracellular ligand binding domain
  - Transmembrane dimerisation domain
  - Intracellular tyrosine kinase domain.
- 185 Kd protein.

HER-2/neu gene amplification & expression

Addition of Herceptin significantly improves overall survival

HER receptor dimer transmembrane signal transduction pathway

Addition of Herceptin significantly improves overall survival

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Herceptin Clinical Trial Program in Breast Cancer

- 1985
- 1990
- 1991
- 1992
- 1993
- 1994
- 1995
- 1996
- 1997
- 1998

- Phase I: 1992-1993
- Phase II: 1994-1995
- Phase III: 1996-1998

- Herceptin in advanced breast cancer 1998
- Herceptin in early breast cancer 2003
- Herceptin in gastric cancer 2011
- Lapatinib, Cetuximab, TDM1 etc.

Accurate determination of “HER2 status” is now pivotal to the management of early breast and gastric cancers.

Error prone HER2 testing:

- Central Herceptest
- IHC Errors:
  - 19% small vs central
  - 3.6% large vs central
  - 5.8% Central vs FISH
  - Central testing is not 100% accurate
  - FISH errors
  - 23.1% small vs central
  - 3.6% large vs central
  - 5% Central vs NSABP
  - Paik et al, JNCI 94:852-4. 2002
- FISH+ 1 1 0 7
- 26.4% discrepancy between local (Mixed IHC methods) and central IHC, 36% with central FISH.
  - Roche et al, JNCI 94:855-7 2002
  - n = 119

ASCO Guidelines Jan 2007

“Approximately 20% of current HER2 testing may be inaccurate.”

“Such a disorganised practice and high rate of inaccuracy – is not acceptable”


Suggests one in every five laboratory tests is incorrect.

Accuracy is paramount:

- "A precise definition of accuracy is how close the measured values are to a supposed true value"
  - Which system most accurately determines HER2 status?
  - Which is least error prone in routine clinical practice?

- "Accurate determination of HER2 status must not be viewed exclusively in terms of benefit from antiHER2 therapy... upstream or downstream anomalies that render the interaction with trastuzumab ineffective.


Concordance does not equal accuracy!

Results Test A
- 90% accurate
Results Test B
- 90% accurate
Concordance will be 81%

If TEST A = 100% accurate
& TEST B = 80% accurate
Concordance will be 80%

Concordant results

Concordance does not equate to accuracy.

Ca 80% of HER2 FISH/IHC results are concordant.
What is the most accurate assay for detection of HER2 overexpression?

- “A precise definition of accuracy is how close the measured values are to a supposed true value”
- Studies with External “gold standard” are rare:
  - FISH vs standard & IHC vs standard.

**HER2 in breast cancer: QIHC – 2 populations**

Two separate populations of breast cancer wto HER2
Robertson et al, Con Res 56:0823-30; 1996.
Overexpressing cancers are gene amplified:

**Accuracy of HER2 tests**

<table>
<thead>
<tr>
<th>Test</th>
<th>Pathvysion</th>
<th>Herceptest</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precision</td>
<td>0.93</td>
<td>0.84</td>
</tr>
<tr>
<td>Kappa</td>
<td>0.74</td>
<td>0.97</td>
</tr>
<tr>
<td>Consistency</td>
<td>99%</td>
<td>64%</td>
</tr>
<tr>
<td>Accuracy</td>
<td>99.2%</td>
<td>93.2%</td>
</tr>
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</table>

When compared with analysis of “true values” for HER2 estimated on frozen tissues, FISH is a more accurate and reproducible diagnostic assay.

**Real world HER2 testing:**

- Can accuracy be maintained in "real world" diagnostic laboratories?
- Data from central versus local laboratories?
- Data from External Quality Assurance schemes
  - (UK NEQAS/US CAP)
  - Independent assessment of performance
  - Standardised samples
- Assess performance over time and track changes
  - Improvements/declines in performance
  - Offer corrective action.
Central laboratories: Methods matter?

<table>
<thead>
<tr>
<th>Local Test</th>
<th>N</th>
<th>Sens</th>
<th>Spec</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>All IHC</td>
<td>1457</td>
<td>0.91</td>
<td>0.75</td>
<td>0.79 (0.77-0.81)</td>
</tr>
<tr>
<td>Herceptest</td>
<td>862</td>
<td>0.92</td>
<td>0.73</td>
<td>0.79 (0.76-0.81)</td>
</tr>
<tr>
<td>Ventana</td>
<td>153</td>
<td>0.94</td>
<td>0.71</td>
<td>0.78 (0.69-0.85)</td>
</tr>
<tr>
<td>FISH</td>
<td>131</td>
<td>0.96</td>
<td>0.90</td>
<td>0.92 (0.80-0.96)</td>
</tr>
</tbody>
</table>

*Press M.F. et al, CCR 11:6598-6607, 2005*

Choice of method explains 6.5-13% of "errors" 
Size of lab may play a role ...
Choice of method has a critical impact on accuracy – high levels of inaccuracy may persist

External Quality Assurance Schemes

- "Real world" testing involves multiple hospitals/laboratories.
- Data from EQA schemes covers large numbers of centres, individually assessed:
  - Can identify optimal and sub-optimal performance – and correct it.
  - UK NEQAS –
    - www.ukneqasicc.ucl.ac.uk/neqasicc.shtml
  - US CAP - proficiency testing survey.
    - Tubbs et al, Archives of Pathology & Laboratory Medicine 126:803-8, 2002
- More on NEQAS tomorrow....

Chromosome 17: is dual colour FISH/ISH important?

Alleles of chromosomes 17 are common in breast cancer. Presence of one hybridization (FISH) purple or orange signal can be assessed in situ at the tissue level and related to the patient's clinical outcome. An increase of multiple FISH probes for the chromosome (ISH) alpha and beta probe sets the HER2 gene were evaluated and found to be significantly higher than those of the FISH probes. The method allows for a precise identification of breast cancer. A high proportion (90%) of breast cancer cases with a high level of gene amplification is possible to be identified. 17q11.2qter identified as an important feature of breast cancer.

- Watters, Going Cooke & Bartlett, 2002 BCRT 77:109-114
- What is CEP17 abnormality?
  - How to define on tissue sections?
  - True frequency and impact on HER2 diagnosis?
  - Does HER2 copy reflect amplification?

Copy false pos/negs
HER2 copy and amplification

- 4% of cases with 3 HER2 copies are gene amplified – monosomy chromosome 17
- 19% of cases with 7 HER2 copies are NOT amplified – Aneusomy or abnormal chromosome 17
- 6.0-7.6% of cases are misdiagnosed by excluding chromosome 17.
- In most cases this causes overtreatment with Herceptin.
- Chromosome 17 must be included for at least 50% of breast cancers.

Chromogenic ISH

Chromogenic ISH multiple chromogens:
- Silver – SISH
- Gold – GOLDFISH
- “CISH” Red/Green etc
All are “chromogenic” assays.
CISH “has the advantage of not requiring fluorescence microscopy.”
CISH “would have to demonstrate a similar high level of accuracy and interlaboratory reproducibility in order to compete effectively with FISH as a diagnostic assay.”

Key papers:

Summary Ventana Silver CISH Ring study.

- Overall concordance = 96.0%
  - 45 cases per lab = 6 laboratories = 270 cases
  - One lab “failed” 95% concordance threshold.
- Intraobserver variation:
  - HER2 CISH=5.9% (all labs) – FISH=4.3% (1 lab)
  - RATIO CISH 8.0% (all labs) – FISH=4.2% (1 lab)

DAKO duoCISH method: (Histopath 2010)

- DuoCISH
  - N =167, concordance 100%.

CISH applied to cores and resection specimens:
Concordance 98%+
Modern Pathology 2012: 1-18
NEQAS ISH Pass rates FISH vs CISH

CISH versus FISH

- Extremely high agreement between current dual colour CISH and FISH methods.
  - Enzymatic development can lead to over/under development.
  - Play extremely close attention to internal QC – signals in normal cells.
- EQA data suggests use of CISH appropriate
  - Some challenges exist – see NEQAS talk tomorrow.
- Recommendation – following careful central validation use with caution.

HER2 Gene Amplification in Breast Cancer

A Rogues’ Gallery of Challenging Diagnostic Cases: UKNEQAS Interpretation Guidelines and Research Recommendations

- ca 90% of FISH/ISH cases are “simple” to interpret.
- There are a subset of “challenging” cases
  - Collectively around 10% of all cases.
- These are “rogues”
  - Clinical evidence for the treatment of these rogues is lacking due to their rarity.
- In order to make treatment recommendations there is a need to “map” these cases to what is known
  - Further research is needed for some groups
    - Starczynski et al American Journal of Clinical Pathology 2012; 137: 595-605

ROGUE Cases and HER2 HETEROGENEITY

HER2 “Rogues”

- Audit of UK Birmingham Heartlands Hospital HER2 ISH cases 2008-2009:
  - Referred due to IHC2+ or other unusual staining patterns.
- 1787 cases audited – 171 (9.6%) exhibited unusual ISH staining patterns.
- Examples isolated reviewed by 9 UK NEQAS ISH reference centres (9)
  - Suggested reporting and research objectives.
“Simple” Heterogeneity

- 2 discrete amplified areas
- Either in close proximity or in different fields.

HER2 HETEROGENEITY AUDIT (CASES)

1. Cases referred to UK Birmingham Heartlands Hospital over 1 year
   - 2+ and equivocal IHC - 1051 eligible cases
2. Cases referred to UK Glasgow reference centre
   - 1657 eligible cases – IHC 0-3+
3. International TEAM pathology Study cases (with outcome)
   - 3753 eligible cases
   - Total cases included in audit: 6461

RESULTS ALL CASES

- Of 6461 eligible cases 910 (14.1%) were amplified as defined by UK guidelines (HER2/CEP17 ratio >2.00).
  - Of these 103 would be defined as "borderline" by the 2007 ASCO/CAP guidelines (ratio between 2.0-2.20).
  - 2166 cases (33.5%) exhibited between 5-50% of cells with a HER2/CEP17 ratio of greater than 2.20 and would under new CAP guidelines be regarded as exhibiting "heterogeneous amplification".
- The CAP guidelines identified over 45% of cases as either amplified or with "heterogeneous amplification".

TEAM PATHOLOGY OUTCOME

- Solid black line <5% cells >2.20 (n = 2481)
- Green line 5-29.9% (n = 1078)
- Blue line 30-49.9% (n = 97)
- Magenta line (N=91)
- Red line 75-99.9% (N=159)
- Dotted black line 100% (N=110).

CAP Guidelines on Heterogeneity

"HER2 genetic heterogeneity (GH) exists if there are more than 5% but less than 50% of infiltrating tumor cells with a ratio higher than 2.2. For example, if 20 cells are counted and at least one cell is identified with a HER2/CEP17 signal ratio higher than 2.2, this specimen contains GH.

- Genetic Heterogeneity in HER2 Testing in Breast Cancer: Panel Summary and Guidelines
  - Vance et al: Arch Pathol Lab Med—Vol 133, April 2009
- What proportion of cases would then be defined as exhibiting heterogeneous amplification?
- Is this a valid definition? Do you use it?
- What impact is there on either outcome or Herceptin response?
  - THERE IS NO DATA TO SUPPORT THIS GUIDELINE
- PERFORM AUDIT linked to OUTCOME.


- Conclusion: Using the new CAP panel guidelines for HER2 "heterogeneous amplification" in an audit of 2920 cases identified 955 (32.7%) cases of heterogeneous amplification, and raised the frequency of "HER2 amplification/heterogeneous amplification" to 44% of cases evaluated. Heterogeneous amplification of the HER2 oncogene is a real and challenging diagnostic finding. Evidence relating to the prognostic impact and in particular response to HER2 therapies is currently lacking for these cases. Guidelines should reflect this and seek to gather such evidence before implementing changes to diagnostic practice.

• The entire slide should be scanned before counting
  – areas of apparent heterogeneity should be identified during this scan and/or by reference to an IHC stained slide.
• The number of chromosome 17 (CEP17) and HER2 signals should be counted in 20 to 60 non-overlapping invasive cancer cell nuclei
  – using at least three distinct tumour fields. If there is evidence of heterogeneity between fields (or less frequently within fields) additional cells (at least 20 per field) and/or fields (up to 6) should be counted.
• The HER2/CEP17 ratio should be calculated for each field individually.
  – Where the mean HER2/CEP17 ratio in any field is 2.00 or greater, the tumour should be regarded as amplified.

**HER2 Gene Amplification in Breast Cancer**

A Rogues’ Gallery of Challenging Diagnostic Cases: UKNEQAS Interpretation Guidelines and Research Recommendations

- ca 90% of FISH/ISH cases are “simple” to interpret.
- How should we report cases with?
  - Different patterns of heterogeneity
    - It’s not always simple
  - Loss of either CEP17 or HER2 signals
  - “co-amplification”
  - NB guidelines are “opinion led” and identify key research questions – is there a right answer?
  - Starczynski et al American Journal of Clinical Pathology 2012; 137: 595-605

<table>
<thead>
<tr>
<th>Distribution of “common” rogues</th>
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<tbody>
<tr>
<td>Percent Rogues</td>
</tr>
<tr>
<td>Hot AMP</td>
</tr>
<tr>
<td>CEP17 loss</td>
</tr>
<tr>
<td>HER2 loss</td>
</tr>
<tr>
<td>CoAMP</td>
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</table>

**Guiding principles:**

1. **For unusual cases** there is no likelihood of a robust evidence base that will determine treatment efficacy with HER2-directed therapies.
2. **Report what you see:** Provide a detailed, adequate description of the ISH/IHC staining pattern.
3. **Reach a conclusion:** Is there sufficient evidence of amplification/overexpression of HER2?
   - Existing treatments are all validated for HER2 overexpressing/amplified cases
4. **Recognise HER2 status is not the final arbiter of clinical decisions**
   - Age, ER, Pgk, Grade, Nodal status, size, Ki67, comorbidity etc etc all play a part
5. **Identify ways to improve “certainty”** – research recommendations

**Current evidence does not support using the existence of small numbers of apparently amplified cells within an individual tumor field to identify heterogeneous amplification.**


**Base funding for OICR is provided by the Government of Ontario through the Ministry of Economic Development and Innovation**
“Simple” Heterogeneity

- 2 discrete areas
  - Either: close proximity or different fields.
- Report “heterogeneous amplification” per UK guidelines.
- Provide ratio for each area scored.

“Complex” Heterogeneity intermixed amplified and non-amplified cells.

Reporting guideline

- Case B amplified and nonamplified cells appear intermingled across the field. Some cells appear normal and some clearly amplified, but it is impossible to distinguish 2 separate fields.
  - Scoring Recommendation.—Score 60 tumor cells across 3 areas, recording results for amplified and nonamplified cells on the same reporting sheet.
  - Interpretation.—This tumor contains intermixed breast cancer clones.
- Reporting:—Report as a case exhibiting potential “intermixed heterogeneous amplification,”
  - Mean HER2/CEP17 ratio for all cells counted
  - Also report percentage of amplified cells (with ratios >2.00).

“Complex” Heterogeneity: research recommendation.

Loss of CEP17 or HER2 signals

- Almost 5% of cases (50% of “rogues”) show loss of either CEP17 or HER2 signals.
- In most cases this does not impact on diagnosis (amplified or not).
- For some cases with loss of CEP17 and duplication of HER2 interpretation may impact treatment.
Deletion of Chromosome 17?

Amplification with loss of CEP17?

- HER2 copy number 5.75 (s.d. 0.9)
- CEP17 copy number 1.0
- Ratio HER2/CEP17: 5.75

a) IF: Loss of non-amplified chr17 = True amplification
b) IF: Loss of CEP17 signal on duplicated chromosomes = false positive

Amplification with loss of CEP17?

- Interpretation.—This is an amplified tumor by HER2 ratio (HER2/CEP17) representing a reduction to monosomy of chromosome 17 with HER2 amplification on the retained chromosome.
- Scoring
- Recommendation.—No evidence scoring additional cells will alter diagnosis.
- Reporting: HER2 gene–amplified - report ratio.
  - Append note of abnormality of CEP17 counts.

Complete loss of Chromosome 17:

- Total loss of all CEP17 signals in the tumor cells; note CEP17 in normal cells
  - HER2 copy number is 6.8 copies/cell (SD,1.78).
- Scoring: Score HER2 in tumor cells
- Interpretation.—Research suggests copy number is an extremely poor guide of HER2 gene amplification.
- Reporting:—Report the case as amplified on the basis of HER2 gene copy number alone (only if ≥6 copies of HER2/cell). Include note to explain the loss of the CEP17 signal in tumor cells.
Loss of CEP17 or HER2 signals
Research recommendation
• HER2 amplified cases with “monosomy 17” may have reduced response to Herceptin
• Need to discriminate between loss of whole chromosome/region and focal loss of CEP17/HER2.
• CGH or MLPA assays would provide insight into specific mechanisms underlying ISH patterns.
• Outcome data unlikely.

HER2/CEP17 “co-amplification”
• Accounts for ca 1-2% of all ISH cases.
• High copy number of both HER2 and CEP17
• Reduced HER2/CEP17 ratio to around 1
• Signals may co-localise

HER2 CEP17 “co-amplification”
• Signals “co-localise”: HER2 copy 24.3, CEP17 23.4 RATIO HER2/CEP 1.04!

Interpretation and reporting
• Interpretation: HER2/CEP17 ratio = 1: high HER2/CEP17.
  – Opinion is that these rare cases should be interpreted as amplified.
  – Preliminary evidence suggests patients with extended amplicons may exhibit reduced response (again metastatic disease)
• Scoring: Score HER2 and CEP17 signals under single-color filters, taking care to match cells within fields to ensure accurate counting of red and green signals.
  – Counting additional cells is not likely to alter the diagnosis.
• Reporting: Amplified with centromere co-amplification
  – Based on HER2 copy and associated balanced CEP17 copy number, plus note co-localization of HER2/CEP17 signals in the report.

Co-Amplified or not?
• High HER2 and CEP17
• Non-overlapping discrete signals.
• Amplified, followed by telomeric non-amplified or deleted targets
• All targets amplified
• Complex patterns
Co-Amplified or not?
• HER2/CEP17 ratio = not amplified
  • HER2 and CEP17 signals high.
    – HER2 >6 often, not uniformly, associated with HER2 amplification.
    – Balanced increase in HER2 and CEP17 copy numbers
      • unlikely to reflect multiple intact copies of chromosome 17.
      • significant room for error in over or under-interpreting FISH results.
      • No clear evidence (colocalized signals) that HER2/CEP17 are coamplified.
    • Difficult to assume this represents true HER2 amplification.
• Reporting: Not amplified comment on the high HER2 and CEP17 copy numbers should be included in the report.
  – Area of greatest discussion/disagreement between experts
  – When is Co-AMP present?
    • 4+4 signals, 5+5, etc? What about 10+10 discrete signals.

Co-amplification of CEP17/ HER2
Research recommendation
• CGH or MLPA assays would provide insight into specific mechanisms underlying ISH patterns.
• Outcome data unlikely.
• All research recommendations require collaborative efforts to identify sufficient cases
  – 15% of breast cancers are HER2 amplified
  – Rogues = 1-2% of these cases = 0.15-0.3% of all breast cancers
    • Test 15000+ cases to identify 25 rogues with similar ISH pattern!

Rogues: Conclusion
• "Rogues" present significant challenges to diagnosis of HER2 status.
• Further research is essential but very challenging
  • Small numbers of specific case types – multi-institutional collaboration/audit.
• Guidelines can produce debate and synthesise data
  – More likely to improve consistency of diagnosis than accuracy?