Ki-67 Index in Breast Cancer

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**2011 St. Gallen Consensus**

<table>
<thead>
<tr>
<th>Subtype</th>
<th>IHC</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER+ and/or PR+, HER2-, Ki-67 &lt; 14%</td>
<td>Endocrine</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER+ and/or PR+, HER2-, Ki-67 ≥ 14%</td>
<td>Endocrine +/- Cytotoxic</td>
</tr>
<tr>
<td>Luminal B (HER2+)</td>
<td>ER+ and/or PR+, HER2+</td>
<td>Endocrine + Cytotoxic + anti-HER2</td>
</tr>
<tr>
<td>HER2</td>
<td>ER-, PR-, HER2+</td>
<td>Cytotoxic + anti-HER2</td>
</tr>
<tr>
<td>Triple Negative</td>
<td>ER-, PR-, HER2-</td>
<td>Cytotoxic</td>
</tr>
<tr>
<td>Genomic Grade Index</td>
<td>Number of Genes</td>
<td>Functions Studied</td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>-------------------------------------------------------</td>
</tr>
<tr>
<td>OncotypeDx RS Paik et al, 2004</td>
<td>16</td>
<td>Proliferation, ER and HER2 invasion</td>
</tr>
<tr>
<td>70 gene signature van’t Veer et al, 2002</td>
<td>70</td>
<td>Cell cycle, angiogenesis, invasion and metastasis</td>
</tr>
<tr>
<td>76 gene signature Want et al, 2005</td>
<td>76</td>
<td>Cell cycle, proliferation, DNA repair, immune response and apoptosis</td>
</tr>
<tr>
<td>Genomic grade index Sotiriou et al, 2006</td>
<td>97</td>
<td>Cell cycle and proliferation</td>
</tr>
</tbody>
</table>
Ki-67: A nuclear marker expressed in all phases of the cell cycle other than the G0 phase.
Ki-67

- Immunohistochemical marker to evaluate tumor proliferation
- Natural candidate to join “prognostic and predictive biomarker club”
- Suggested as marker for differentiation of luminal A and luminal B tumors (St. Gallen panel, 2011)
- An International study to increase concordance in Ki67 Scoring (2015 Mod Pathol)
Issues in Ki-67 Index by IHC

• How can we standardize the methods for Ki67 analysis?
• What is the optimal point Ki-67 cut-off point for prognosis in breast cancer?
• Concordance between core biopsy and surgical specimen. Which one is more accurate?
• Visual vs. Automated image analysis systems?
How can we standardize methods for Ki67 analysis?
Assessment of Ki-67 in Breast Cancer: Recommendation from the International Ki-67 Breast Cancer Working Group

- Pre-analytical setting
- Analytical setting
- Interpretation and scoring
- Data analysis

J Natl Cancer Inst 2011
Type of fixative

Avoid all but neutral buffered formalin. Others reduce Ki67 staining compared with neutral buffered formalin.

JNCI 2011;103:1656-1664
Antigen retrieval

- Antigen retrieval procedures are required.
- Although Ki-67 IHC is tolerant to a variety of epitope retrieval protocols, protease and low PH methods should be avoided.
- The best evidence supports the use of heat-induced retrieval most frequently by microwave processing.


Microwave-antigen retrieval: the importance of pH of the retrieval solution for MIB-1 staining.

Boon ME.
Leiden Cytology and Pathology Laboratory, The Netherlands.

Abstract
Using microwaves for microscopy almost invariably implies working at elevated temperatures. In microwave studies it became evident that high temperatures do not inevitably damage the tissues. On the contrary very high temperatures can even be beneficial. The most striking example is the 'heat shock' of formalin-fixed paraffin sections in the microwave oven with temperatures higher than 100 degrees C, revealing otherwise hidden epitopes. These microwave applications have triggered a breakthrough in pathology. The pH of the microwave-retrieval solution is decisive for the staining results, particularly for MIB-1. There is no staining of proliferating cells between pH 3.5 and 5.0. Between pH 5.0 and 6.5 we observed an increase in the number of MIB-1-positive stained nuclei. Our findings indicate that in quantitative work it is of extreme importance to standardise the pH of the retrieval solution and to choose pH 6.5 for optimal results.
Primary antibody

MIB1 clone
- The most commonly used mouse anti-human Ki-67 monoclonal antibody
- Consistent and much better performance across a wide range of antibody dilution and conditions, compared to other proliferation markers such as PCNA
- The most widely validated

→ The MIB1 antibody is currently endorsed for Ki-67.

SP6 clone
- Rabbit human Ki-67 monoclonal antibodies
- SP6 clone against Ki-67 appear promising (especially for image analysis) but insufficient data to support routine use at this time.

JNCI 2011;103:1656-1664
J Clin Pathol 2010;63:800-804
Ki67

MIB1
(mouse monoclonal, Zymed)

SP6
(rabbit monoclonal, Neomarkers)
Immunohistochemical assessment of Ki67 with antibodies SP6 and MIB1 in primary breast cancer: a comparison of prognostic value and reproducibility

Ekholm M et al Histolopathology, 2014
Method of reading

- Only nuclear staining is considered positive. Staining intensity is not relevant.
- Scoring should involve the counting of at least 500 malignant invasive cells (and preferably at least 1000 cells) unless a protocol clearly states reasons for fewer being acceptable.
- Image analysis methods for Ki67 remain to be proven for use in clinical practice.

JNCI 2011;103:1656-1664
Area of slides read

• In full sections, at least three high-power (×40 objective) fields should be selected to represent the spectrum of staining seen on initial overview of the whole section.
• For the purpose of prognostic evaluation, the invasive edge of the tumor should be scored.
• If pharmacodynamic comparisons must be between core cuts and sections from the excision, assessment of the latter should be across the whole tumor.
• If there are clear hot spots, data from these should be included in the overall score.

JNCI 2011;103:1656-1664
### Results: Clinico-pathological subtypes by proliferation assessment method (N=474)

<table>
<thead>
<tr>
<th>Assessment Method</th>
<th>Luminal A</th>
<th>Luminal B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67 100</td>
<td>10%</td>
<td>63%</td>
</tr>
<tr>
<td>Ki67-1020 Periphery</td>
<td>24%</td>
<td>49%</td>
</tr>
<tr>
<td>Ki67-1020 Spectrum</td>
<td>34%</td>
<td>39%</td>
</tr>
<tr>
<td>MAI</td>
<td>52%</td>
<td>24%</td>
</tr>
</tbody>
</table>
Cut –off Points ?
The best Ki67 index cut point to distinguish luminal B from luminal A tumors was 13.25%.

In St Gallen 2011 Recommendation;
This cut-point is derived from comparison with gene array data as a prognostic factor. Optimal cut-points in Ki-67 labelling index for prediction of efficacy of endocrine or cytotoxic therapy may vary.

Prognostic significance of Ki-67 index value at the primary breast tumor in recurrent breast cancer

Nishnura R et al MOLECULAR AND CLINICAL ONCOLOGY 2015
Ki-67 prognostic cut-off point for all breast cancer subtypes (multivariate analysis)

<table>
<thead>
<tr>
<th>Cut-off Value</th>
<th>HR</th>
<th>Cumulative Disease-Free Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>10%</td>
<td>1.75</td>
<td></td>
</tr>
<tr>
<td>20%</td>
<td>1.91</td>
<td></td>
</tr>
<tr>
<td>30%</td>
<td>1.75</td>
<td></td>
</tr>
<tr>
<td>40%</td>
<td>1.85</td>
<td></td>
</tr>
<tr>
<td>45%</td>
<td>1.88</td>
<td></td>
</tr>
<tr>
<td>50%</td>
<td>1.78</td>
<td></td>
</tr>
</tbody>
</table>

The best cut-off point with the lowest p-value and highest HR was found at the Ki-67 index of 20%.
Ki67 levels as predictive and prognostic parameters in pretherapeutic breast cancer core biopsies: a translational investigation in neoadjuvant GeparTrio trial.

IHC of 1166 pretherapeutic core biopsies (large sections)

Analysis strategy:
- Systematic cutpoint analysis
- Web-based open source software
  Cutoff Finder [http://molpath.charite.de/cutoff/](http://molpath.charite.de/cutoff/)
- Analysis for tumor subtypes
• pCR: 94 of 95 cutpoints significant
• Three outcome parameters combined: all cutpoints between 10% and 45% Ki67 are significant
• Data derived cutpoint optimization is not possible
• For further analysis: three groups
  – Ki67 low: ≤ 15%
  – Ki67 intermediate: 15.1-35%
  – Ki67 high: >35%
• These cutpoints
  – Can be used for all three endpoints (pCR, DFS, OS).
  – Result in three groups of equal size in the GeparTrio cohort
  – Cutpoint(s) should be defined by the scientific community.

Denkert C et al, Ann Oncol 2013, 24:2786
Concordance between core biopsy and surgical specimen. Which one is more accurate?
Ki67 proliferation in core biopsies versus surgical samples
- a model for neo-adjuvant breast cancer studies

50 consecutive breast cancer cases with both a core biopsy and a surgical sample available, without intervening neo-adjuvant therapy

Romero et al. BMC Cancer 2011
Dichotomization of core biopsies and surgical samples into low and high proliferation as determined by Ki67 evaluation using a 20% cut-off value

<table>
<thead>
<tr>
<th></th>
<th>200 cells</th>
<th></th>
<th>1000 cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 20%</td>
<td>&gt; 20%</td>
<td>Total</td>
<td>&lt; 20%</td>
</tr>
<tr>
<td>Surgical samples</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 20%</td>
<td>19</td>
<td>10</td>
<td>29</td>
<td>29</td>
</tr>
<tr>
<td>&gt; 20%</td>
<td>2</td>
<td>19</td>
<td>21</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>21</td>
<td>29</td>
<td>50</td>
<td>33</td>
</tr>
</tbody>
</table>
## Summary of statistical analysis for 200 and 1000 cancer cells after evaluation for Ki-67 proliferation

<table>
<thead>
<tr>
<th>Statistical test</th>
<th>200 cells</th>
<th></th>
<th>1000 cells</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean difference (95% CI)</td>
<td>P</td>
<td>Mean difference (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td><strong>t-test, linear scale</strong></td>
<td>3.9% * 0.1% - 7.8%</td>
<td>0.046</td>
<td>2.2% * -1.1% - 5.6%</td>
<td>0.19</td>
</tr>
<tr>
<td>t-test, linear scale excluding outlier</td>
<td>4.8% * 1.2% - 8.3%</td>
<td>0.009</td>
<td>3.1% * 0.03% - 6.0%</td>
<td>0.03</td>
</tr>
<tr>
<td>t-test multiplicative scale</td>
<td>0.81 ** 0.65 – 1.01</td>
<td>0.063</td>
<td>0.85 ** 0.67 – 1.08</td>
<td>0.18</td>
</tr>
<tr>
<td>Wilcoxon**</td>
<td>0.03</td>
<td></td>
<td>0.06</td>
<td></td>
</tr>
</tbody>
</table>

Disparate pairs (distribution)

Romero et al. BMC Cancer 2011
Visual vs. Automated
<table>
<thead>
<tr>
<th>군집</th>
<th>개체수</th>
<th>개체수</th>
<th>% 개체수</th>
<th>등계 면적 (Area)</th>
<th>최대 사각형 너비 (Max Bound Width)</th>
<th>Feret 최대 너비 (Feret Max)</th>
<th>Feret 면적 (Feret Area)</th>
<th>회색 평균 밀도 (Density 회색 Mean)</th>
<th>파란색 평균 밀도 (Density 파란색 Mean)</th>
<th>녹색 평균 밀도 (Density 녹색 Mean)</th>
<th>빨간색 평균 밀도 (Density 빨간색 Mean)</th>
</tr>
</thead>
<tbody>
<tr>
<td>군집 1</td>
<td>311</td>
<td>22.1</td>
<td>7.9%</td>
<td>39.5</td>
<td>30.4</td>
<td>30.6</td>
<td>40.6</td>
<td>22.3</td>
<td>15.0</td>
<td>17.2</td>
<td>20.2</td>
</tr>
<tr>
<td>군집 2</td>
<td>1095</td>
<td>77.9</td>
<td>12.1%</td>
<td>60.5</td>
<td>69.6</td>
<td>69.4</td>
<td>59.4</td>
<td>77.7</td>
<td>85.0</td>
<td>82.8</td>
<td>79.8</td>
</tr>
</tbody>
</table>
Comparison of Visual and automated assessment of Ki-67 proliferative activity and their impact on outcome in primary operable invasive ductal breast cancer

- TMA (n=379) immunostained for Ki-67
- Visual and Automated with the Slidepath Tissue IA system
- Visual and automated Ki-67 LI were in excellent agreement (ICCC=0.96, P<0.001)
- Automated Ki-67 LI assessment was inferior in predicting cancer survival in patients with breast cancer, including patients who received Tamoxifen

Mohammed ZMA et al, British Journal of Cancer, 2012
In GEPARTRIO study, Klauschen F et al, sabcs 2012

- Good overall agreement between manual and automated ($r > 0.8$, $p < 0.001$)
- Better performance of manual scoring in survival analysis
An international study to increase concordance in Ki67 scoring

- **Analytical validity:** To what level of reproducibility can pathologists reliably quantify Ki67 staining?

- **Identify sources of inter- and intra-observer variability:**
  - TMA slides, local methods (Phase 1)
  - Web-based calibration
  - TMA slides, after calibration + standard method (Phase 2)

Polley MY et al, JNCI2013 and Mod Pathol 2015
Lessons learned from Phase 1

• Intra-observer consistency good, but interobserver variability problematic

• Cut points not freely transferable – local recalibration against clinical endpoint or reference images is needed

• Although staining method added some variability, the major source of Ki67 differences (besides patient biology) was scoring methods.
  – Estimation vs. Counting
  – Choice of areas to count
  – Invasive Cancer vs. other cells
  – Threshold of brown considered “positive”

Polley MY et al, JNCI, 2013
Lessons learned from calibration

• Labs were “trainable: performance did improve, although improvement not statistically significant, perhaps due to sample size.
• Labs differed on what threshold of “brown” they considered positive.
• Although these data are potentially encouraging, suggesting that it may be possible to standardize scoring Ki-67 among pathology laboratories, clinically important discrepancies persist.
• Before this biomarker could be recommended for clinical use, future research will need to extend this approach to biopsies and whole sections, account for staining variability and link to outcomes.

Polley MY et al, Mod Pathol 2015
WS and TMA of 20 surgically resected cases of invasive ductal carcinomas
(T1-10 : without hot spots, T11-20 : with hot spots)

Chung YR et al, Korean Breast Pathology Ki-67 Study Group, 2014 (not published)
• Inter-observer variability of Ki67 index for direct counting and categorical estimation was relatively high although direct method was slightly better than categorical estimation.

• Tumors with hot spots showed greater inter-observer variability as opposed to those without.

• Restricting the measurement area by constructing TMAs yielded lower inter-observer variability but counting from the hot spot area did not improve inter-observer concordance.

• Categorical estimation can be used for tumors with high Ki-67 index, but it is not suitable for those with low to intermediate Ki-67 index.

Chung YR et al , Korean Breast Pathology Ki-67 Study Group,2014 ( not published)
Take home messages

- The current studies do indicate that Ki-67 index have a valuable role as prognostic and predictive markers.
- Ki-67 is highly influenced by methodological issues and the examined tumor spot.
- Therefore, its limitations should be kept in mind when treatment decisions are made.
- Ki-67 in the range of 14 ~20% could serve as a good prognostic cut-off point in Luminal and non-Luminal breast cancer subtypes.
- Core biopsy Ki-67 values are acceptable, but may underestimate the Luminal tumor aggressiveness.
- There is an good agreement between manual and automated system. It remain to be proven for use in clinical practice.
Thank you!