Digital image analysis and a novel set of cell line samples as aids in the development of a quantitative external quality assessment programme for Ki-67

Fitim Berisha[1], Dawn Wilkinson[1], Lila Zabaglo[1], Suzanne Parry[1] & Andrew Dodson[1, 2]

1. UK NEQAS ICC & ISH, 5 Coldbath Square, London EC1R 5HL UK. 2. Corresponding author: adodson@ukneqasiccish.org

UK NEQAS International Quality Expertise

Layman's Summary

Ki-67 is a protein that can be detected in the nuclei of cells that are dividing to make new copies of themselves, while in contrast, cell that are in a resting (non-dividing) state lack Ki-67 expression.

By measuring the proportion of tumour cells that are expressing Ki-67 using a method called immunohistochemistry (IHC) it is possible to get important information about how quickly the tumour is growing and by implication, how aggressive it is. This 'prognostic information' as it is called can be combined with other clinical features to help the clinician advise the patient in a more informed way when they are deciding on which course of treatment is most appropriate.

For example, in early-stage primary breast cancer (that is cancer that has not spread beyond the breast and local lymph nodes) a significant proportion of tumours that show good prognostic features (including low levels of Ki-67 positive cells) can be successfully treated by a combination of surgery, radiotherapy and endocrine therapy (the latter only works if the tumour is oestrogen receptor positive – more than 80% are). Importantly, there is no need to use chemotherapy, which is the type of therapy that often causes the 'more unpleasant' side-effects we associate with cancer treatments, such as nausea, hair-loss, fatigue and diarrhoea.

It is clearly very important that hospital laboratories measure Ki-67 levels correctly. One way they can help to ensure they are producing good quality results is by comparing their testing output with that of over a hundred other similar laboratories. They can do this by joining our Ki-67 in Breast Cancer Module, which looks at exactly this.

The results of the research that we are presenting here shows how we can give improved feedback to laboratories about the quality of their results by asking them to test specially made cell-line samples that very accurately identify IHC methods that show correct levels of Ki-67 positive cells. And another cell-line that identifies methods that have a tendency to produce false-positive results that are much higher than they should be.

Introduction

Ki-67 is a well-established biomarker of proliferation in breast cancer (BC). However, the value of the prognostic information it provides to the treatment decision making process is hampered by a lack of analytical reproducibility despite much work to improve this [1].

It has been shown that regular participation in external quality assessment (EQA) programmes, such as the ones provided by the UK National External Quality Assessment Scheme for Immunocytochemistry and In-Situ Hybridisation (UK NEQAS ICC & ISH) can substantially improve laboratory performance. And more specifically, it has shown this for other important breast cancer markers, such as oestrogen receptors [2].

UK NEQAS ICC & ISH has previously reported on a Ki-67 in Breast Cancer EQA programme that it has recently established. Currently the test substrates used in the programme are primary BC samples individually showing low (<5%) and high (>20%) proliferation rates. With assessment being by manual observation and digital image analysis (DIA) [3].

We report here on an enhancement to the Ki-67 in Breast Cancer programme with the introduction of specifically designed cell-line preparations, which when analysed by DIA separately provide additional information on method agreement levels and the presence of inappropriate/non-specific staining.

Materials and Methods

A formalin-fixed paraffin embedded (FFPE) cell-line microarray (CMA) was designed and produced in conjunction with Array Sciences LLC (Sausalito, USA).

The CMA was composed of:

- a core taken from a pure population of Sf9 caterpillar cells, which has previously been reported to lack reactivity with antibodies to human Ki-67 [4].

Together with cores of Sf9 cells mixed with four different human BC cell-lines at different proportions:

- 85% BT-20 cells (15% Sf9 cells),
- 75% ZR-75-1 cells (25% Sf9 cells),
- 65% BT-474 cells (35% Sf9 cells),
- 55% BT-483 cells (45% Sf9 cells).

Sections from the CMA were cut and mounted onto glass microscope slides together with sections from a FFPE tonsil sample and two BC samples. One BC showed high (~30%, BC-high) and the second, low proliferation (~5%, BC-low). Unstained sections were distributed to laboratories participating in the Scheme's Ki-67 in Breast Cancer programme.

Laboratories stained the slides for Ki-67 using their routine method. Returned slides were centrally manually assessed for stain quality. Scanned whole-slide images were made using a NanoZoomer S360 (Hamamatsu, Japan) and subjected to DIA using an in-house application developed using Visiopharm software (Visiopharm A/S, Hoersholm, Denmark).

Results

A total of 60 laboratories returned stained slides for analysis (Table 1).

Primary antibody clone & Automation	Count (n)	Proportion (%)
MIB-1 (RTU/Concentrate, Dako)	**33**	**55.0**
BenchMark Ultra (Ventana)	15	25.0
Omnis (Dako)	9	15.0
Bond III (Leica Microsystems)	8	13.3
Autostainer Link 48 (Dako)	1	1.7
30-9 (RTU, Ventana)	**19**	**31.7**
BenchMark Ultra (Ventana)	17	28.3
Omnis (Dako)	2	3.3
SP6 (RTU, Cell Marque)	**2**	**3.3**
Bond III	2	3.3
MM1 (RTU, Leica Biosystems)	**1**	**1.7**
Bond III (Leica Microsystems)	1	1.7
Unknown	**5**	**8.3**
Bond III (Leica Microsystems)	2	3.3
BenchMark Ultra (Ventana)	3	5.0
TOTAL	**60**	

Table 1. Primary antibodies and automated platforms used.
Four different primary antibody clones were reported as been used among the 55 laboratories that returned methodology data. The most commonly used primary was MIB-1 (33, 55.0%).

Four different automated staining platforms were used (all laboratories returned data for this aspect of their method). The BenchMark Ultra, supplied by Ventana was the platform with the largest number of users (35, 58.3%)

RTU = ready to use.

Results (continued)

Part I. Use of the pure Sf9 caterpillar cell-line to identify false-positive staining.

Analysis of Ki-67 scores obtained on the Sf9 core identified two distinct groups:

- The first group (n = 38, 63.3%) showed no Ki-67 positivity (DIA score = 0) or had DIA scores of <5% (Mean score = 1.1%, 95% Confidence Intervals (CIs): 0.2 - 1.9%).
- The second group (n = 22, 36.7%) displayed a step-change in DIA scores (Mean = 49.9%, 95% CIs: 33.2 - 66.7%).

The means of the two groups were significantly different (P<0.0001).

When Ki-67 scores for each of the two BC samples were analysed separately, similar results were obtained for both the BC-low and the BC-high sample sets compared to that seen for the group as whole.

Quality scores generated by manual assessment did not differ significantly between the two groups. But it must be taken into account that these manual assessments had been on the tonsil and the two BC samples and had not considered the cell-line staining. When the Sf9 cores were visually examined aberrant nuclear staining was clearly visible, and non-specific nuclear staining could also be identified in the matched BC tissue samples (Figure 1).

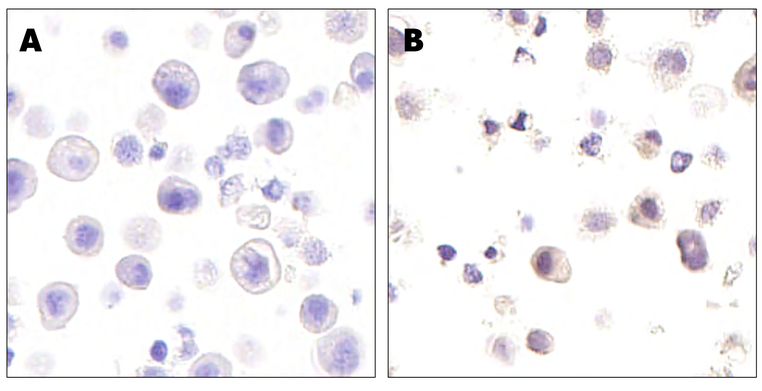

Figure 1. Illustrations of nuclear staining in Sf9 cells.
The staining shown in **Panel A** is from a preparation of pure Sf9 cells in which the DIA score was 0. The cell and nuclear morphology is well preserved, and the blue colour of the nuclear counterstain is clearly seen.

Panel B illustrates an example of false-positive nuclear staining which was identified by the DIA, cellular and nuclear detail is poorly preserved. There is a brown hue in most nuceli. Both preparations x40 original magnification.

Part II. Use of breast cancer cell lines as an aid to the quantification of Ki-67 staining.

Correlation of Ki-67 scores between the four BC cell line cores and each of the BC tissue samples was examined using Pearson's correlation statistics.

The *r* statistic range was:

- 0.61 - 0.69 in comparisons between BC cell-line cores and the BC-high sample
- 0.43 - 0.56 when Ki-67 scores for BC cell-line cores were compared with the BC-low sample.

In each case BT-483 showed the highest correlation score and BT-20 the lowest.

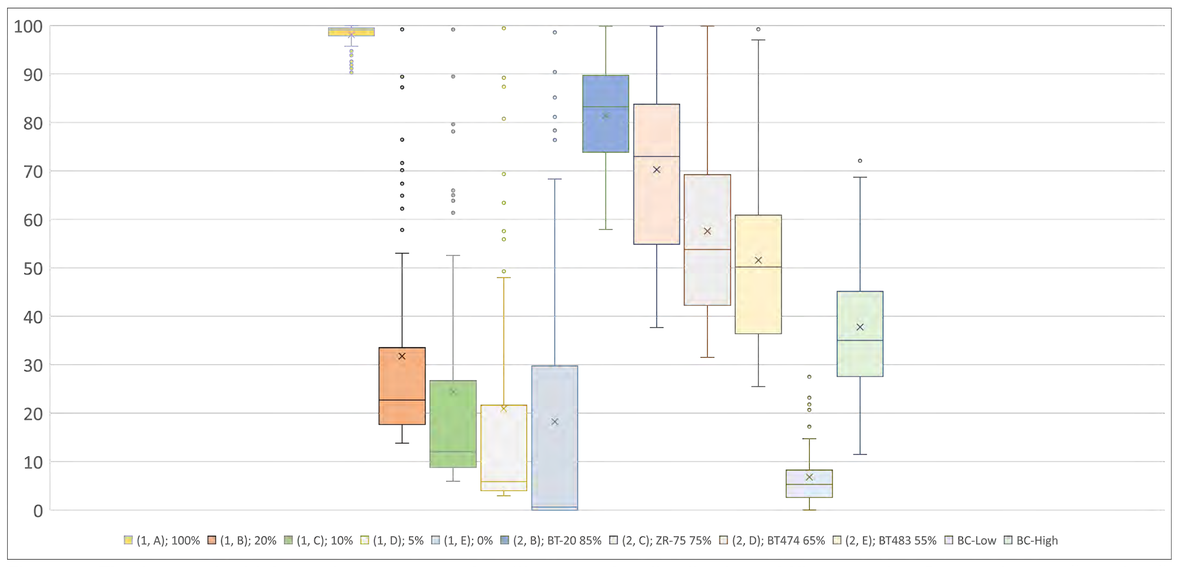

Figure 3. The box and whiskers plots were used to illustrate the distribution of Ki-67 results. Boxes show 25th and 75th percentiles, whiskers show 5th and 95th percentile. The line indicates the median and cross the mean result.

	(1, A) 100%	(1, B) 20%	(1, C) 10%	(1, D) 5%	(1, E) 0%	(2, B) BT-20 85%	(2, C) ZR-75 75%	(2, D) BT474 65%	(2, E) BT483 55%	BC-Low	BC-High
Minimum	95.0	13.8	6.0	3.0	0.0	57.9	37.7	31.6	25.5	0.0	11.5
1st Quartile	97.9	17.7	8.8	4.0	0.0	73.9	54.9	42.3	36.5	2.7	27.6
Median	99.1	22.8	12.1	6.0	0.7	83.3	73.0	53.8	50.2	5.4	35.1
3rd Quartile	99.5	33.6	26.8	21.7	29.8	89.8	83.8	69.2	60.9	8.3	45.1
Maximum	100.0	53.0	52.6	48.0	68.4	99.9	99.9	99.9	97.1	14.8	68.7
Mean	98.1	31.9	24.5	21.0	18.3	81.4	70.3	57.6	51.6	6.9	37.9

Table 2. Descriptive statistics for the CMA cores and the BC samples.

Conclusions

We have developed a sensitive indicator of non-specific nuclear staining in IHC preparations stained for Ki-67 by using a pure population of Sf9 caterpillar cells, which identifies the presence of the artefact quantifiably.

Cores made from Sf9/BC cell line mixtures (especially BT-483) produce Ki-67 scores which correlate with those obtained in breast cancer samples at a similar level to those achieved between tonsil and BC samples; this is true for both high and the low proliferation ranges.

Cell line mixtures can be adjusted to show Ki-67 scores in the clinically relevant ranges, and they do not show the inherent biological variations seen in tissue controls such as tonsil.

References
1. Nielsen TO, et al. Assessment of Ki67 in Breast Cancer: Updated Recommendations From the IKBC Working Group. JNCI. 2021;113:808-819.
2. Parry S, et al. UK NEQAS ICC & ISH Oestrogen Receptor Module Results. Eur Congress Pathol. 2018; abstract.
3. Parry S, et al. UK NEQAS ICC & ISH Ki-67 Data Reveal Differences in Performance of Primary Antibody Clones. AIMM. 2021;29:86-94.
4. Aung TN, et al. A new tool for technical standardization of the Ki67 immunohistochemical assay. Mod Pathol. 2021;34(7):1261-1270.

UK Interdisciplinary Breast Cancer Symposium

Poster number: 63. Birmingham, 2024