HPV/p16 Analyte Control

Utility review and ring study results

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**Collaboration:**

- Dr Max Robinson at Newcastle University a leading pathologist in head and neck cancer.
- Keith Miller, United Kingdom National External Quality Assurance Scheme for ICC and ISH (UKNEQAS).

**Drivers:**

- Clinical trials – large volume assessments. No consistency of control material across all cases when using tissue as same slide controls.
- EQA – varying quality of assays in HPV ISH and p16 despite “standardisation”.
- Unmet needs assessment:
  
  Testing varies by country. HPV ISH vs p16. Assessment of cervical carcinoma often different to oral and oropharyngeal squamous cell carcinoma. Significant demand in USA.
• While vaccines are reducing rates of HPV infection and associated cancers this is primarily in females.
• Oral cancer is still the 6th leading cancer by incidence globally (WHO).
• Virtually all cases of cervical cancer are caused by HPV infection, with HPV 16 & 18 detected in 70%. 1,2
• HPV 16 is responsible for around 85% of anal cancers and HPV 16 & 18 account for approximately 50% of vaginal, vulval and penile cancers. 3
• Within the last 20 years, the incidence of HPV-related oropharyngeal squamous cell carcinoma (OPSCC) has increased, particularly among men.
• HPV 16 has been identified in around 50% of OPSCC in the US. 4
• It has been estimated that, by 2020, HPV will cause more OPSCC than cervical cancers in the US. 5
• p16 positivity is a useful surrogate marker of oncogenic HPV infection. p16 negative oral cancer are typically caused by tobacco.
• HPV-related OPSCC tend to have a better prognosis.
• There is emerging evidence to suggest that HPV positive patients may benefit from de-escalated treatments.

HistoCyte Laboratories Ltd developed: High-Risk Human Papilloma Virus (16, 18) Control Slides for same slide use in:

- HPV DNA in situ hybridization
- E6/E7 mRNA in situ hybridisation
- p16 immunohistochemistry

The following slides show typical staining achieved with the HPV/p16 Analyte ControlDR using:

- Ventana/Roche
  - CINtec® p16 Histology assay. Ready to use (RTU) antibody.
  - INFORM III HPV ISH assay
- ACDBio RNAScope E6/E7 mRNA assay
p16 immunohistochemistry

- p16 protein expression strongly associated with HPV infections.
- Cell (A) negative (no HPV)
- Cells (B) and (C) have high homogeneous expression throughout cell population.
- Cell (D) has a high heterogeneous expression (Cell D).

Ventana/Riche CINtec® p16 Histology assay
HPV DNA in situ Hybridization

- HPV DNA in-situ hybridisation.
- Cell (A) is HPV negative.
- Cell (B) has very low HPV16 gene copies.
- Cell (C) has medium HPV18 gene copies.
- Cell (D) has high HPV16 gene copies.

Ventana/Roche INFORM III HPV ISH assay
• mRNA in-situ hybridisation for E6/E7. ACD RNAscope

• Cell (A) is negative, though there is occasional background staining, it is not a genuine signal.

• Cell (B) is a low positive cell line, which reflects the low HPV gene copies.

• Cells (C) and (D) have high levels of HPV mRNA.

ACDBio RNAscope E6/E7 mRNA assay
As part of verification and validation, HistoCyte Laboratories Ltd assessed multiple batches over an extended period to determine stability and reproducibility. All testing was done with Roche/Ventana assays.

To determine their utility in the market, HistoCyte Laboratories Ltd conducted a “Ring Study” across 8 clinical sites:
- 5 tested both HPV and p16
- 8 tested for p16

All HPV ISH assays were with Ventana/Roche:
- X7 sites used p16 from Ventana/Roche:
  - 4/7 on Ventana Benchmark
  - 2/7 on Leica Bond
  - 1/7 on Dako Autostainer.
- X1 site used Santa Cruz Ab on Ventana Benchmark.

The results are summarised in the following slide.
Excessive cytoplasmic staining in Cell D

Clean cytoplasm in Cell D

All slides were anonymised and scored independently by two assessors.
## Ring study results: HPV

### Summary of results from ring study: ISH

<table>
<thead>
<tr>
<th>Site No.</th>
<th>1</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platform</td>
<td>Ventana/Roche</td>
<td>Ventana/Roche</td>
<td>Ventana/Roche</td>
<td>Ventana/Roche</td>
<td>Ventana/Roche</td>
</tr>
<tr>
<td>Assay</td>
<td>HPV ISH</td>
<td>HPV ISH</td>
<td>HPV ISH</td>
<td>HPV ISH</td>
<td>HPV ISH</td>
</tr>
<tr>
<td>Cell A</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cell B</td>
<td>&lt;5% (1-2 sigs)</td>
<td>&lt;5% (1-2 sigs)</td>
<td>&lt;5% (1-2 sigs)</td>
<td>&lt;5% (1-2 sigs)</td>
<td>&lt;5% (1-2 sigs)</td>
</tr>
<tr>
<td>Cell C</td>
<td>&gt;50% (&gt;2 sigs)</td>
<td>&gt;50% (&gt;2 sigs)</td>
<td>&gt;60% (&gt;2 sigs)</td>
<td>&gt;80% (&gt;2 sigs)</td>
<td>&gt;60% (&gt;2 sigs)</td>
</tr>
<tr>
<td>Cell D</td>
<td>&gt;80% (sigs in clts)</td>
<td>&gt;90% (sigs in clts)</td>
<td>&gt;99% (sigs in clts)</td>
<td>&gt;99% (sigs in clts)</td>
<td>&gt;80% (sigs in clts)</td>
</tr>
</tbody>
</table>

* Cells appear over digested, morphology disrupted to some degree. Still interpretable. Clts: clusters.

All slides were anonymised and scored independently by two assessors.
The most striking result was the difference in sites that had both used the same assay and yet cell line D was generally providing two different results.

1. Strong nuclear staining in 30-60% of cells with cytoplasmic staining.
2. Strong nuclear staining 30-50% of cells with no cytoplasmic staining.

The ISH results were consistent, cell C had signals in 50-80% of cells. The higher percentage correlated with excessive digestion demonstrated by damaged cell architecture (site 7).

To determine the consistency of the p16 scoring done manually, the slides were scanned and assessed using Visiopharm image analysis. Performed by Visiopharm we were able to demonstrate consistency in the scoring (see next two slides) regardless of the excessive cytoplasm
Case 5 – “HCL standard” No staining in the cytoplasm of cell D. As seen in our development.
Site 6 – Typical of strong staining and excessive staining in the cytoplasm of cell D.

**HCL Assessment**

<table>
<thead>
<tr>
<th>Ventana/Roche Assay</th>
<th>p16 IHC</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cell A</strong></td>
<td>0 (blush)</td>
</tr>
<tr>
<td><strong>Cell B</strong></td>
<td>5+ N/C &gt;99%</td>
</tr>
<tr>
<td><strong>Cell C</strong></td>
<td>5+ N/C &gt;99%</td>
</tr>
<tr>
<td><strong>Cell D</strong></td>
<td>4-5+ N/C 50-60% C Blush 2+</td>
</tr>
</tbody>
</table>

**VisioPharm Analysis**

- **Cells Counted**: 14,737
- **Positive Percentage**: 62%
Observations from HPV in ISH

• Only 5 of the sites performed HPV ISH – All Roche Inform.
• All state they’re using the “standard” protocol from Roche.

Site 5

Site 6

Site 7

• Over digested Cell line D at Sites 6 and 7. Site 5 is a typical result.
• Site 6 protease step is 24 minutes compared to 8 minutes at Site 7 and 5.
• Denaturation is “standard” 2 hours with CC1.
• Sometimes the reasons for the differences are less obvious. In each of these cases the assay has worked as signal is very clearly seen, however, the cell integrity was clearly compromised.
Why differences in staining

While each site had assays that provided appropriate results in terms of determining p16 expression and gene copy numbers, the quality clearly varied.

Variations in protocols
• UltraView versus OptiView
• Amplification versus no-amplification
• Antibody incubation times
• RTU dilution
• Automation

Some variation is due to platform and/or associated chemistries or antibody
The difference was solved on further enquiry with the sites. It appears that some laboratories dilute the Roche RTU clone.

Those sites that do dilute lose the cytoplasmic staining.

It was done because the RTU staining was excessive and they could get satisfactory staining by diluting the RTU.

The risk is that the product is used outside of the manufacturers recommendations.

It appears from this small study that cell line D can determine how this antibody is being used. Importantly both results are correct in as much as the cell line is p16 positive. The ultimate use of the antibody is defined by the laboratory and not by these controls.
HPV ISH sensitivity

• Cell line B is a well characterised cell line with 1-2 signals per cell.
• Affected by plane in which the cell is cut and sensitivity of the assay:

  ![Diagram showing cell cuts and signals]

  X2 Signals
  No Signals

• Can be easily overlooked!
• In US field trials they were called negative. Slide review showed they were positive.
• Created three core version of the product which was considered easier to use in the USA.
Summary of HPV/p16

• Created a very good QA/QC tool. Through phenotype and genotype the cells are able to demonstrate a number of things:
  • IHC: Antibody usage and suitable protocol
  • ISH: Slide treatment/digestion and efficacy of the probes
• Allows some degree of trouble shooting. In future we hope to determine specific issues based on cell performance.
• Standardised appears anything but!
• p16 may become more important as a Companion Diagnostic as a surrogate marker for targeting of CDK4 in a variety of cancers\textsuperscript{1-3}.
• For more information: info@histocyte.com
