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Cover illustration: Visualisation of immunoglobulin genes by fluorescence in situ hybridisation in phytohaemagglutinin stimulated lymphocytes. Triple labelling of metaphase chromosomes with DAPI (blue), a painting library for chromosome 22 (red) and a plasmid Cλ probe (green). (See: Carvalho *et al.* In situ visualisation of immunoglobulin genes in normal and malignant lymphoid cells. *J Clin Pathol: Mol Pathol* 1995;48:M158–64.)

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Human papilloma virus detection by in situ hybridisation signal amplification based on biotinylated tyramine deposition

The greatest advantage of non-isotopic in situ hybridisation (NISH) over PCR amplification, for the detection of human papilloma virus (HPV), is a combination of morphological preservation with cellular localisation. Hence, the description of a novel NISH signal amplification method,[1] using catalysed reporter deposition (CARD-ISH) is especially pertinent for HPV DNA detection in paraffin wax embedded sections. This is particularly useful for the detection of low copy number HPV DNA below the threshold of conventional NISH methods. Using the CARD-ISH amplification system, Poddighe *et al*[1] demonstrate 1–3 integrated copies of HPV DNA in SiHa cells producing distinct intranuclear punctate/dot signals. This is undoubtedly far more sensitive than the usual detection threshold of viral copies per cell with NISH.[2]

However, Poddighe *et al* also used their amplification system for detecting about 500 integrated copies of HPV16 DNA in CaSKi cells and 20–50 integrated copies of HPV18 DNA in HeLa cells. The resultant amplified signal with CARD-ISH in both these human cervical carcinoma cell lines is a blotchy, lumpy intranuclear signal. This latter signal destroys the distinctive punctate/dot signal that can easily be demonstrated with conventional NISH methodology.[2] The significance of the punctate/dot-like signal (type 2 NISH signal pattern) is that it denotes an integrated physical state of HPV DNA in cervical neoplasia[3]; adding a third advantage of NISH over PCR, other than morphological preservation and cellular localisation. Hence, it would appear that CARD-ISH would destroy this valuable signal pattern in a cell containing more than 20–50 integrated viral copies. The type 2 NISH signal pattern, already validated by three independent laboratories,[3-5] is a simple technique for the detection of integrated HPV DNA in cervical neoplasia. The use of the CARD-ISH system would therefore be useful only for producing and preserving this signal pattern in low copy number HPV DNA.

Another unusual phenomenon illustrated by Poddighe *et al*[1] is a cervical lesion with HPV DNA demonstrated by NISH positive/PCR negative. Although the highly sensitive general primer (GP5+ and GP6+) mediated PCR[6] directed towards the L1 open reading frame was used, it nevertheless failed to demonstrate HPV DNA. This phenomenon of NISH positive/PCR negative HPV DNA in clinical samples has been demonstrated previously.[7] Conventional NISH requires approximately 12 viral copies per cell for detection of HPV DNA in paraffin sections.[2] In contrast, it has been estimated that PCR requires at least 400 HPV DNA viral copies in a paraffin wax embedded clinical sample for amplification of the virus.[8] This extends further the advantages of NISH over PCR. The alternative explanation for the NISH positive/PCR negative cervical lesion in the Poddighe study may be related to the integration of HPV16 DNA in this case with disruption of L1 ORF, preventing amplification with the GP5+/GP6+ PCR system that targets the L1 gene. Although the E2 gene is consistently disrupted with HPV DNA integration into host chromosome, the L1 gene may also occasionally be interrupted.[9] In such instances, the use of PCR systems directed towards E6 may be more useful for the demonstration of the HPV DNA.

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1 Poddighe PJ, Bulten J, Kerstens HMJ, Robben JCM, Melchers WJG, Hanselaar AGJM. Human papilloma virus detection by in situ hybridisation signal amplification based on biotinylated tyramine deposition. *J Clin Pathol: Mol Pathol* 1996;**49**:M340–4.
2 Herrington CS, Graham AK, McGee JO'D. Interphase cytogenetics III: increased sensitivity and flexibility of digoxigenin labelled DNA probes for HPV detection in cervical biopsies and cell lines. *J Clin Pathol* 1991;**44**:33–80.
3 Cooper K, Herrington CS, Stickland JE, Evans MF, McGee J O'D. Episomal and integrated human papillomavirus in cervical neoplasia shown by non-isotopic in situ hybridisation. *J Clin Pathol* 1991;**44**:990–6.
4 Kristiansen E, Jenkin A, Holm R. Coexistence of episomal and integrated HPV 16 DNA in squamous cell carcinoma of the cervix. *J Clin Pathol* 1994;**47**:253–6.
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6 De Roda Husman A-M, Walboomers JMM, van den Brule AJC, Meijer Chris JLM, Snijders PJF. The use of general primers GP5 and GPO elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. *J Gen Virol* 1995;**76**:1057–62.
7 Grayson W, Taylor L, Cooper K. Detection of integrated high risk HPV in adenoid cystic carcinoma of the uterine cervix. *J Clin Pathol* 1996;**49**:1–5.
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9 Choo K-B, Pan C-C, Han S-H. Integration of HPV type 16 into cellular DNA of cervical carcinoma: preferential deletion of the E2 gene and invariable retention of the long control region and the E6/E7 open reading frames. *Virology* 1987;**161**:259–61.

Dr Poddighe comments:
The three human cervical cell lines CaSKi (> 500 integrated copies of HPV 16 DNA), HeLa (20–50 integrated copies of HPV18 DNA) and SiHa (1–3 integrated copies of HPV16 DNA) were used to determine the sensitivity, specificity, and the possibility of routine diagnostic application of our CARD-ISH technique compared with conventional NISH methodology. Our conclusion from these results is that a few integrated HPV DNA copies (1–3 copies per nucleus in SiHa cells) can easily be recognised with CARD-ISH. Cooper and Taylor are correct in their comment that the resultant amplified signal with CARD-ISH destroys the distinctive punctate/dot signals of HPV DNA in nuclei of the cell lines CaSKi and HeLa. However, in our 1995 paper concerning the CARD-ISH technology[1] we showed that it was possible to obtain distinct punctate/dot ISH signals in nuclei by varying the biotinylated tyramine concentration, the incubation time or both. In the later paper concerning HPV detection using CARD-ISH[2] we wanted to show that our new CARD-ISH approach enables routine study of cervical premalignant lesions and carcinomas for infection with (low copy numbers of) HPV DNA in combination with morphological preservation. False negative results because of insufficient sensitivity of conventional NISH methods will be minimised to almost zero. In fact, the use of the entire HPV genome as a probe in our CARD-ISH methodology is superior to PCR as the latter approach may lead to false negative results (and therefore misdiagnosis of an HPV infection) resulting from disruption, deletion or mutation of primer annealing sites, as suggested by Cooper and Taylor. Of course, the intranuclear or cellular localisation of individual HPV copies in cervical neoplasia can be studied by conventional NISH methodology only when at least 12 viral copies per cell are present in paraffin sections.[3]

1 Kerstens HMJ, Poddighe PJ, Hanselaar AGJM. A novel in situ hybridization signal amplification method based on the deposition of biotinylated tyramine. *J Histochem Cytochem* 1995;**43**:347–52.
2 Poddighe PJ, Bulten J, Kerstens HMJ, Robben JCM, Melchers WJG, Hanselaar AGJM. Human papilloma virus detection by in situ hybridization signal amplification based on biotinylated tyramine deposition. *J Clin Pathol: Mol Pathol* 1996;**49**:M340–4.
3 Herrington CS, Graham AK, McGee JO'D. Interphase cytogenetics III: increased sensitivity and flexibility of digoxigenin labelled DNA probes for HPV detection in cervical biopsies and cell lines. *J Clin Pathol* 1991;**44**:33–80.

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