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Human papilloma virus is associated with breast cancer

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BACKGROUND: There is increasing evidence that high-risk human papilloma virus (HPV) is involved in cancers in addition to cervical cancer. For example, it is generally accepted that HPV has a role in a significant proportion of head and neck tumours, and it has long been hypothesised that hormone dependent oncogenic viruses, such as HPV may have causal roles in some human breast cancers. A number of reports have identified HPV DNA in breast tissue and breast cancer specimens, but these rely on standard polymerase chain reaction (PCR), which is criticised for its propensity for contamination.

METHODS: We have used two different technologies, *in situ* and standard PCR (with sequencing), and histology based on light microscopy.

RESULTS: We unambiguously demonstrate the presence of high-risk HPV in the cells of breast cancer specimens and breast cancer cell lines. In addition, we also show that the oncogenic characteristics of HPV associated breast cancer are very similar to HPV-associated cervical cancer. Specifically, that putative koilocytes are present in some HPV associated breast cancers.

INTERPRETATION: The above observations indicate a likely causal role for high-risk HPV in human breast cancer and offer the possibility of primary prevention of some breast cancers by vaccination against HPV.

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Although the association of human papilloma virus (HPV) with cervical cancer, and head and neck cancers is well established, the involvement of the virus in breast cancer is more controversial. Previous studies have demonstrated the presence of HPV high-risk types 16, 18 and 33 in breast cancer specimens from diverse populations around the world: Italy, Norway, China, Japan, USA, Austria, Brazil, Australia, Taiwan, Turkey, Greece, Korea, Mexico, Hungary and Syria (Lonardo *et al*, 1992; Hennig *et al*, 1999; Yu *et al*, 1999; Liu *et al*, 2001; Damin *et al*, 2004; Widschwendter *et al*, 2004; de Villiers *et al*, 2005; Kan *et al*, 2005; Tsai *et al*, 2005; Gumus *et al*, 2006; Kroupis *et al*, 2006; Lawson *et al*, 2006; Choi *et al*, 2007; Akil *et al*, 2008; Khan *et al*, 2008; Kulka *et al*, 2008; Mendizabal-Ruiz *et al*, 2009). The prevalence of HPV positive breast cancer in these studies was reported to vary from 4% in Mexican to 86% in American women. In all studies, high-risk HPV was found in tumour tissue only and not in surrounding normal tissue, with the exception of the study from Turkey, in which the virus was also detected in normal tissue but at a lower level than in the cancer (Gumus *et al*, 2006). Although the route of transmission for the virus has not been determined, women positive for both breast and cervical cancers were found to be infected with the same HPV type in both tumours (Hennig *et al*, 1999; Widschwendter *et al*, 2004).

The controversy surrounding the role of HPV in breast cancer may be because of the difficulty that has been encountered in detecting the virus in breast specimens, in contrast to the relative ease of detection in cervical cancers (Lindel *et al*, 2007; Khan *et al*, 2008). Indeed, in a previous study from our group, we demonstrated that it was necessary to use SYBR Green I (Molecular Probes, Carlsbad, CA, USA) for polymerase chain reaction (PCR) detection of virus in breast cancer in DNA extracted from breast tissue (Kan *et al*, 2005). This is considered to be because of the fact that a considerable proportion of breast cancer specimens is non-cancerous and that the levels of virus are low in breast cancer. One solution to the detection of such low levels of HPV is the use of *in situ* PCR.

The oncogenic mechanisms by which HPV induces cervical cancer have been intensively studied (zur Hausen, 2002). In this study, we have used HPV-associated cervical cancer as a model. High-risk HPV encodes a series of proteins, designated as early (E1 – E7) or late (L1 and L2). Although all of the viral proteins have a role in viral replication, only a small number of the viral early proteins have a role in cellular transformation. Key to transformation are the E6 and E7 oncoproteins, which work in concert to disrupt cell-cycle regulation, inhibit apoptosis and stimulate cell-cycle progression by binding/inhibiting the p53 and $p110^{RB}$ tumour suppressor genes, respectively. In addition, and relevant to this study, HPV E5 and E6 act early in transformation (before integration) and are known to disrupt cytokeratin causing perinuclear cytoplasmic clearing and nuclear enlargement, which leads to the appearance of a koilocyte (Krawczyk *et al*, 2008; Thomison *et al*, 2008).

If HPV is oncogenic in human breast cancer, we hypothesise that high-risk HPV should be present in (some) human breast cancers and in some normal and pre-cancerous tissue (although at a lower proportion), and that koilocytosis should be apparent in

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HPV-associated breast cancers, and that HPV will be detected in some proportion of breast cancer cell lines.

In this paper we report on the presence of HPV in breast cancer cell lines, in the nuclei of cells within the cancerous regions of breast cancer specimens and correlate the presence of HPV with the histopathological features of HPV-induced transformation.

MATERIALS and METHODS

Archival specimens

Unselected formalin-fixed breast cancer specimens and non-cancer specimens from women who had breast reduction surgery, were analysed using *in situ* PCR. All the specimens were from women living in Australia. A total of 28 breast cancer specimens and 28 unselected non-cancerous breast specimens were suitable for use in this *in situ* PCR study. Fifteen of the fixed specimens were ductal carcinoma *in situ* (dcis) and 13 were predominantly invasive ductal carcinomas (idc).

Breast cancer cell lines

Standard PCR techniques were used to determine if HPV DNA genetic material was present in the human breast cancer cell lines: MCF-7, T47D, BT-549, HBL-100, Hs578.T, MDA-MB-453, MDA-MB-468, MDA-MB-175-VII and SK-BR-3.

In situ PCR

Four-micron thick sections of breast tumour tissue were cut and placed on silanised slides. Positive controls (known virus-positive cervical cancer tissue), and negative controls (known virus-negative tissue), and controls omitting DIG-11-dUTP (to confirm incorporation of DIG label), omitting primers (to confirm the signal was the result of specific amplification rather than self priming of degraded tissues), or omitting Taq polymerase, were also undertaken for each specimen. The primers used for HPV *in situ* PCR for the L1 region of HPV-16 and HPV-18 were forward (5′-GCMCAGGGWCATAAYAATGG-3′) and reverse (5′-CGTCCM ARRGGAWACTGATC-3′). The outcomes were assessed by the staining of the DIG label in the tissues by light microscopy.

A number of specimens gave a false-positive signal when the primers were omitted. The use of exo-zap, initial filling in with Klenow, and doing two rounds of PCR – the first with no DIG-11-dUTP and the second with DIG-11-dUTP – gave the same false-positive results with these particular specimens. Consequently, we carried out a (no-primer) negative control for each sample at the same time as the *in situ* screening PCR and eliminated the specimens that gave false-positive colour signals. Any specimens that produced a signal in the *in situ* PCR negative controls (omission of the DNA primers and separately, omission of the Taq polymerase) were eliminated from the study. False positives indicated that the DNA was self-priming and were unsuitable for *in situ* work (this is probably because of fragmented DNA, caused by formalin fixation, acting as primers). In addition, tissues were screened by *in situ* PCR for β-globin to confirm their suitability for this study.

To confirm the validity of the outcomes of *in situ* PCR analyses, DNA was extracted from the same formalin-fixed specimens and standard PCR analyses were conducted. The methods used are outlined below. The products were sequenced and the identity of the sequences was determined using the BLAST alignment system.

Standard PCR

Genomic DNA preparation Previously described protocols were used to extract genomic DNA from the breast cancer specimens (Dawkins *et al*, 1993). The DNA quality was tested by the amplification of a 268-bp fragment of the β-globin gene using HotStarTaq DNA polymerase (Qiagen, Dusseldorf, Germany) and primers G073 (5′-GAAGAGCCAAGGACAGGTAC-3′) and G074 (5′-CAACTTCATCCACGTTCACC-3′). The cycling conditions were 95°C for 9 min; followed by 35 cycles of 95°C, 30 s; 55°C, 30 s; 72°C, 1 min; and a final extension at 72°C, 10 min. The amplified products were visualised on 2% agarose gel.

Screening for HPV sequences Standard PCR was carried out in a total volume of 50 μl using HotStarTaq DNA polymerase (Qiagen) with the following conditions: 95°C for 9 min; followed by 35 cycles of 95°C, 30 s; 55°C, 40 s; 72°C, 40 s; and a final extension at 72°C, 10 min. Cell lines were screened using the MY/GP HPV consensus primers as described by Kroupis *et al* (2006), omission of the DNA template was used as a negative control. Both MY and GP primers sets amplify the L1 region of the HPV genome and are used as a preliminary step for HPV screening. The amplified products were visualised on a 1.5% agarose gel and sequenced to confirm the type of HPV. The PCR was independently repeated for each sample.

Histology

The presence of koilocytosis in the fixed breast cancer series was assessed by light microscopy with koilocyte positive cervical cancer specimens used for comparison. Koilocytes were best characterised by (Koss and Durfee, 1956) the presence of large cells with relatively small, but irregular and hyperchromatic nuclei surrounded by clear and transparent cytoplasm. Koilocytosis is restricted to the replicating basal cells and multinucleation is common in these cells. The histological features of HPV positive breast tumours are similar to cervical koilocytosis and have previously been reported by de Villiers *et al* (2005).

RESULTS

Breast cancer cell lines

In this study, we demonstrate the application of *in situ* PCR to identify HPV sequences within breast cancer cells. We initially screened nine breast cancer cell lines (as listed in Materials and Methods) for HPV *in situ* PCR. High-risk HPV gene sequences were identified in two of the cell lines (MDA-MB-175-VII and SK-BR-3) of the nine lines tested. *In situ* PCR demonstrated that HPV DNA was confined within the nucleus of the cells (Figure 1). The presence and type (HPV type 18) was confirmed by automated sequencing (Figure 2) of the PCR products shown in Figure 3.

Identification of HPV DNA sequences in formalin-fixed breast cancer specimens by *in situ* PCR and sequencing

Having established the method and demonstrated the presence of HPV in breast cancer cell lines, we screened a series of fixed breast cancer and normal breast tissue specimens using *in situ* PCR. As discussed in Materials and Methods section, we eliminated those samples, which potentially could give false-positive HPV results, identified by positive *in situ* PCR without primers. An unknown number of these eliminated specimens would have been true positives. Accordingly, the data cannot be used to make estimates of prevalence of the presence of these viruses.

High-risk HPV DNA sequences were identified in the nuclei of breast cancer epithelial cells in 5 (39%) of 13 dcis and 3 (23%) of 13 idc breast cancer specimens (Figure 4, summarised in Table 1). Unexpectedly, we saw HPV containing cells in the surrounding normal tissue of some samples (Figure 4D). In all 3 (18%) of 17 normal breast specimens (from breast reduction surgery) were HPV positive by *in situ* PCR (Table 1). The presence of HPV in

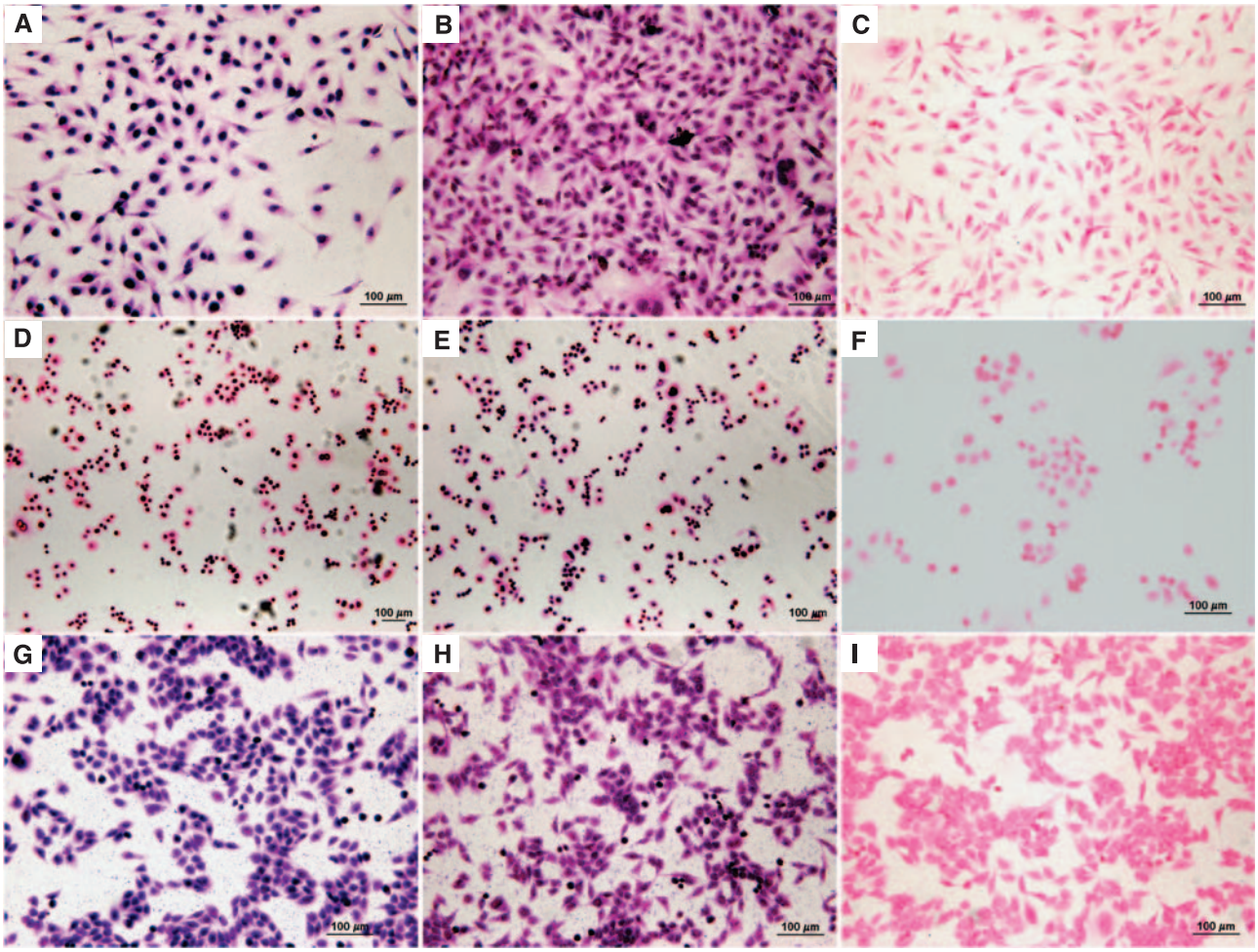

Figure 1 Human papilloma virus (HPV) *in situ* polymerase chain reaction (PCR) of breast cancer cell lines. (**A**–**C**) Cell line MBA-MB-175V11, (**D**–**F**) cell lines BR-SK3 and (**G**–**I**) cell lines HeLa (HPV-18 containing cervical cancer cell line: positive control). (**A**, **D** and **G**) *In situ* PCR with HPV E6 primers. (**B**, **E** and **H**) *In situ* PCR with HPV L1 primers. (**C**, **F** and **I**) No primer (negative) *in situ* PCR control.

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Spec 2.TTTAACAATATGTGCTTCTACACAGTCTCCTGTACCTGGGCAATATGATGCTACCAAATTTAAGCAGTATAGTAGACATGTT
Spec 4.TTTAACAATATGTGCTTCTACACAGTCTCCTGTACCTAGTCAATATGATGCTACCAAATTTAAGCAGTATAGCAGACATGTT
Spec 5.TTTAACAATATGTGCTTCTACACAGTCTCCTGTACCTGGGCAATATGATGCTACCAAATTTAAGCAGTATAGCAGACATGTT
Spec 6.TTTAACAATATGTGCTTCTACACAGTCACCTGTACCTGGGCAATATGATGCTACCAAATTTAAGCAGTATAGCAGACATGAT

SK-BR3 TTTTACA-TATGTGCTTCTACACAGTCTCCTGTACCTGGGCAATATGATGCTACCAAATTTAAGCAGTATAGCAGACATGTT
MDA-MB-175ACA TTATGTGCTTCTACACAGTCTCCTGTACCTGGGCAATATGATGCTACCAAATTTGAGCAGTATAGCATACATGTT
HeLa   TTTAACAATATGTGCTTCTACACAGTCTCCTGTACCTGGGCAATATGATGCTACCAAATTTAAGCAGTATAGCAGACATGTT
       *** ***  ****************** ********* * ********************** ********** * ***** *

Spec 7.AAATATGTCATTATGTGCTGCCATATCTACTTCAGAAACTACATATAAAAATACTAACTTTAAGGAGTACCTACGACATGGG
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Figure 2 Polymerase chain reaction (PCR) product nucleotide sequences of human papilloma virus (HPV)-positive patient samples and breast cancer cell lines. Four HPV-positive breast cancer samples were identified in Figure 4 below (specimens 2, 4, 5 and 6) and two breast cancer cell lines (SK-BR3 and MDA-MB-175) were identified as HPV type 18, whereas one breast cancer specimen (specimen 7) was identified as HPV type 16. Minor sequence variations were observed in three samples (specimens 2, 4 and 6) and in both cell lines (SK-BR and MDA-MB-175) when matched against reference sequence HPV-18 positive HeLa. There is no sequence variation in specimen 7 when matched against HPV-type 16 genome (accession FJ006723).

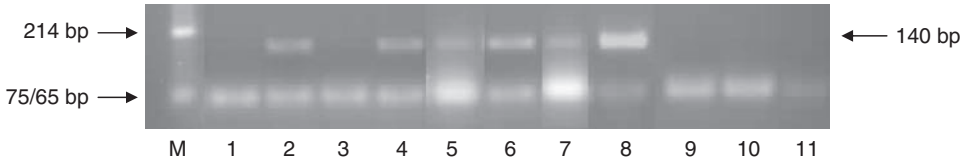

Figure 3 Human papilloma virus (HPV) screen of patient samples using MY and GP primers. Lane M is the Puc/Hinf ladder marker. Lanes 1–7 are patient samples (breast cancer specimens 1–7). Lane 8 is HeLa DNA as the positive control. Lanes 9–11 are negative controls (water in place of DNA in reaction). Lanes 2 (specimen 2), 4 (specimen 4), 5 (specimen 5), 6 (specimen 6) and 7 (specimen 7) show positive bands of 140 bp. Both samples in lanes 1 (specimen 1) and 3 (specimen 3) are negative for HPV.

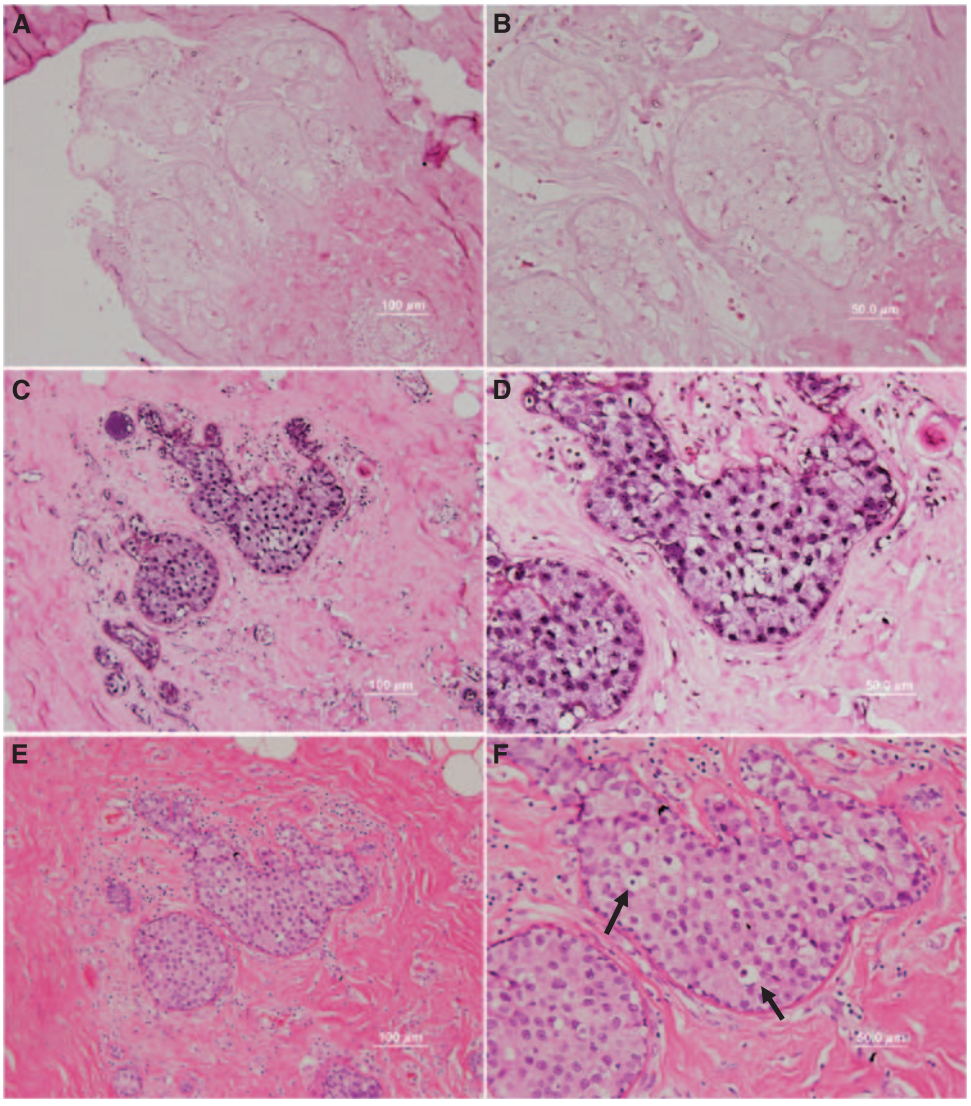

Figure 4 Human papilloma virus (HPV) in cancer cells of ductal carcinoma *in situ* breast cancer demonstrated by *in situ* polymerase chain reaction (PCR) (same specimen in all panels). (**A** and **B**) No primer *in situ* PCR control (× 20 and × 40 objective, respectively), (**C** and **D**) HPV L1 primer *in situ* PCR (× 20 and × 40 objective, respectively), Dark purple stain in panels **A**–**D** indicated amplification of HPV L1 sequences by *in situ* PCR. (**E** and **F**) Haematoxylin and eosin stain (× 20, × 40 objective, respectively). The appearance of koilocytes in the HPV-18 containing cells shown in panel **F** (selected koilocytes shown by arrows) is indicated by the clearing of the cytoplasm and condensed, hyperchromatic nuclei.

Table 1 *In situ* PCR screening for HPV in fixed breast tissue specimens and breast cancer cell lines

	Number analysed by *in situ* PCR	Number HPV positive (%)	HPV-type 18 (by PCR/sequencing) (%)	HPV-type 16 (by PCR/sequencing) (%)	Number HPV positive (%)
Ductal carcinoma *in situ*	13	5 (39)	5		5 (39)
Invasive ductal carcinoma	13	3 (21)	2	1	3 (21)
Normal breast tissue	17	3 (18)	3		3 (18)
Breast cancer lines	9	2 (22)	2		2 (22)

Abbreviations: HPV = human papilloma virus; PCR = polymerase chain reaction.

normal breast tissues is consistent with the requirement for HPV infection in the breast tissue before HPV-induced tumourigenic transformation of a single clone.

Seven breast cancer specimens and three normal specimens showed false-positive outcomes when the PCR primer was omitted from the *in situ* PCR analysis. These specimens were not considered further.

Confirmation of *in situ* PCR analyses

Deoxyribonucleic acid was extracted from the HPV-positive specimens and analysed by standard PCR (Figure 3) to confirm the findings based on *in situ* PCR and to type the HPV by sequencing (Figure 2). Not all gels and sequences are shown in Figures 2 and 3. The outcome was confirmed in all the three HPV-

positive normal breast specimens. The outcome was confirmed in five of the eight HPV-positive breast cancer specimens. Minor sequence variations indicate that contamination was unlikely.

All HPVs were type 18 except for one specimen in which HPV was type 16. The identification of two HPV types is a further indication that contamination is unlikely. Known HPV-positive (cervical cancer specimens and HeLa cervical cancer cultured cells) and negative (no PCR primers) were used as controls. All controls gave the expected outcomes.

HPV-associated morphological characteristics

The features of koilocytosis were observed in 18 (66%) of 28 breast cancer specimens (Figure 4). Koilocyte-like cells were also observed in some HPV-positive normal breast tissue specimens. The koilocyte-like cells were all HPV positive as shown by *in situ* PCR. These features are very similar to koilocytosis present in HPV-positive cervical cancer.

DISCUSSION

In this report, we have confirmed the presence of HPV in the nuclei of cells in breast cancer tissue. The use of *in situ* PCR localises the HPV to the nuclei of cells within the cancerous tissue, and substantially decreases the possibility that detection of HPV in breast cancer is a contamination. In addition, the detection of HPV was consistent with the cell morphology and standard PCR/sequencing results.

The detection of HPV in breast cancer is consistent with 15 (of 17) previous publications reporting the presence of HPV in breast cancer world-wide with a prevalence of ranging from 4–86% (Lawson *et al*, 2006). Given such a high proportion of studies reporting HPV in breast cancer and breast tissue, the question is why some (2 of 17) studies report that HPV is not present in breast cancer. Potential explanations include difficulties in detection due to low viral load and low frequency of HPV in breast cancers in some populations. The *in situ* PCR results demonstrate just how much non-cancerous tissue is present in breast cancer specimens, which may explain the difficulty in detection of HPV by standard PCR (upon DNA extraction from the whole tissue sample).

The observation that HPV type 18 was by far the most common type in these Australian breast cancers is meaningful as most breast cancers can be regarded as originating from breast-milk epithelial cells (despite the historically misleading terminology of 'ductal' breast carcinomas). Therefore, most breast cancers are 'glandular'. HPV type 18 has an affinity or tropism to glandular as compared with squamous epithelial cells (Clifford and Franceschi, 2008).

It is important to note that we report the presence on high-risk HPV in normal breast tissue. This supports the previous report of HPV in normal tissue in Turkish breast samples (Gumus *et al*, 2006). The presence of HPV in some normal breast samples (normal breast and normal surrounding breast tissue as shown in Figure 4) is consistent with a tumourigenic role for HPV in some breast cancers. If HPV has a causal role in breast cancer, it is reasonable to expect that this would be an early event, similarly with cervical cancerogenesis, and that HPV would be found in at least some normal tissue. Furthermore, it is expected that the presence of high-risk HPV would not be sufficient for full tumourigenic transformation and further changes would accumulate over time in a step-wise manner.

Significance of these findings

Although the proportion of HPV-positive breast cancer specimens in this study is higher than HPV-positive normal breast tissues, the overall number of specimens is small and definitive conclusions cannot be made. However, given the propensity for HPV to oncogenically transform human epithelial cells, including breast epithelial cells (Band *et al*, 1990), plus the unambiguous evidence that high-risk HPVs are present in the nuclei of breast cancer cells and in breast cancer cultured cell lines, and that HPV-positive koilocytes are present on many normal and breast cancer specimens, suggests that HPV may have a causal role in many breast cancers.

Establishing an oncogenic role for HPV in some breast cancers leads to the possibility of primary prevention of some breast cancers by vaccination against HPV, as current vaccines are known to be effective against HPV types 16 and 18 (Rambout *et al*, 2007).

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