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Detection of cutaneous HPV types 4 and 24 DNA sequences in breast carcinoma in Singaporean women of Asian ancestry

KEITH ONG*, EVELYN SIEW-CHUAN KOAY†‡ AND THOMAS CHOUDARY PUTTI‡

*Department of Pathology, National University Hospital, †Molecular Diagnosis Centre, Department of Laboratory Medicine, National University Hospital, ‡Department of Pathology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

Summary

Aim: To date, there have been conflicting data on the prevalence of HPV in primary breast carcinoma, with a median prevalence of 35%. We believe that the low prevalence reported could be due to use of inappropriate amplification primers and methods of detection. We designed a study to detect and reflect more accurately the incidence of both mucosal and cutaneous HPV types in breast carcinoma among Singaporean women.

Methods: In our study, we used two different molecular techniques, both of which involved a polymerase chain reaction (PCR) amplification step for any HPV DNA found in the breast cancer tissues. The first method targeted primarily the mucosal HPV types, and the second, primarily the cutaneous HPV types. Formalin-fixed, paraffin-embedded breast cancers were used for the studies. The first method involved the GP5+/GP6+ primers for PCR amplification and a commercially available HPV DNA genotyping chip for detection. The subsequent studies involved the use of the FAB 59/64 primers for amplification, followed by DNA sequencing and comparison to the NCBI GenBank database for the detection of all possible HPV types.

Results: With the first technique, all 92 breast cancers tested gave negative results for HPV DNA, suggesting the absence of HPV types in breast cancers. Using the second method, we detected HPV sequences in 32/92 (35%) samples, of which 28 were shown to be HPV-4, one was HPV-24, two had mixed HPV types and one had an indeterminate HPV sequence that did not match any of the HPV sequences deposited in the GenBank database.

Conclusions: These results were consistent with our hypothesis that the true incidence of HPV in breast carcinoma is much higher than those reported to date, and that this is probably due to the limited sensitivity of the molecular techniques used in earlier studies.

Key words: Breast, HPV, DNA, carcinoma, virology.

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INTRODUCTION

Papillomaviruses are small, non-enveloped viruses with 55 nm diameter icosahedral capsids that contain double-stranded DNA genomes of approximately 8000 base pairs (bp). Approximately 200 different human papillomaviruses (HPVs)[1] have now been characterised. HPVs specifically infect squamous epithelia, and their infections have been shown to be associated with a number of neoplastic conditions,[1,2] ranging from common warts to carcinomas of the larynx, cervix and oesophagus. HPVs can be classified into mucosal and cutaneous HPVs. Within each of these HPV groups, individual viruses are designated high risk or low risk, according to the propensity for malignant progression of the lesions that they cause. Most HPVs are low risk and produce localised benign warts that do not undergo malignant progression, even if left untreated. Among the cutaneous HPV types, HPV-5 and HPV-8 may be classified as high risk, as they are associated with the development of epidermodysplasia verruciformis (EV),[1] a hereditary skin disorder characterised by widespread skin eruptions of flat-to-papillomatous, wart-like lesions and reddish-brown pigmented plaques on the trunk, the hands, the upper and lower extremities, and the face. Low risk mucosal HPVs such as HPV-6 and HPV-11 cause genital warts (condyloma accuminata), whereas the high risk HPVs cause squamous intraepithelial lesions that can progress to invasive squamous cell carcinoma. The vast majority of human cervical cancers are associated with high risk HPV infections. HPV-16 is by far the most prevalent mucosal high risk HPV type, followed by HPV-18, HPV-31, and others. The evidences of these associations come from the studies that prove the oncogenic ability of the E6/E7 HPV gene[1] and the very high prevalence (>99%) of HPVs reported in cervical carcinoma.[3,4] Within the family of mucosal HPVs, the low risk/high risk classification parallels the transforming potential of the respective viral genomes in cell culture and transgenic mouse models. Hence, much of the molecular research conducted to date has focused on the analysis of the transforming activities of mucosal high risk HPVs that are associated with cervical cancer. The extent of molecular research on cutaneous HPVs pales by comparison.

The identification of HPVs in human breast tumours and the immortalisation of normal human breast cells by HPV types 16 and 18[5] have aroused the suspicion that HPVs might have a role in the pathogenesis of breast carcinoma. However, unlike in cervical carcinoma, the reported prevalence of HPV DNA in breast carcinoma ranged from 0 to 86%. We reviewed the existing literature and noted that in most of the previous studies, the primer sets used were designed primarily for mucosal HPV types. We hypothesised

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that the low prevalence reported could be due to usage of inappropriate primers for amplification, leading to sub-optimal detection rates. The aim of our study was to accurately detect any mucosal and cutaneous HPV types in breast carcinoma, if infection was present. In order to do so, we used two different molecular techniques, one utilising the GP5+/GP6+ primers[6] that had been designed primarily to detect the mucosal HPV types, and the other employing the FAB 59/64 primers[7] designed primarily to detect the cutaneous HPV types.

MATERIALS AND METHODS

Materials

Representative sections of paraffinised breast tumour tissues were supplied by two of the authors (KO and TCP) for the HPV analysis. At the outset, 115 cases were selected from routine mastectomy or lumpectomy specimens removed in the course of treatment for carcinoma of the breast. Of these, 23 cases were rejected, due to failure to obtain patient consent or due to pre-operative chemotherapy for the breast carcinoma. Of the remaining 92 cases, 21 cases were randomly selected from the 2003 cohorts of breast carcinoma and 71 cases from the 2004 cohorts of breast carcinoma, diagnosed at the National University Hospital, Singapore. The patients, all female, ranged in age from 19 to 86 years and their ethnic composition was 74 Chinese, 11 Malay and 7 Indian. None of them had documented cervical carcinoma. An overview of patient and tumour characteristics is shown in Table 1.

DNA extraction from paraffin embedded tissue

DNA extraction was carried out using the DNeasy Tissue Kit (Cat #69506; Qiagen, Germany). The appropriate areas of the paraffin-embedded tissue section, containing tumour cells, were removed from the microscopic slide with scalpels, and transferred into a 1.5 mL Eppendorf tube containing 1.20 mL of xylene to deparaffinise the tissues. The tube was centrifuged at full speed for 5 min at room temperature, and after removing the supernatant, 1.20 mL of absolute ethanol was added to the pellet, and the contents were centrifuged at full speed for 5 min at room temperature to remove the residual xylene. This wash step was repeated to ensure complete removal of all traces of xylene and paraffin.

The washed cell pellet was incubated at 37°C for 5–10 min to allow the ethanol to evaporate, resuspended with 180 μL of Buffer ATL and 20 μL of Proteinase K, and incubated at 55°C in a shaking water bath until the cells were completely lysed. After lysis, 200 μL of Buffer AL was added and mixed well by vortexing, followed by a further incubation at 70°C for 10 min. Next, 200 μL of ethanol (96–100%) was added to the sample, mixed thoroughly by vortexing, and the mixture transferred onto a DNeasy mini column, and centrifuged at 6000 × *g* (8000 rpm). The flow-through from the previous and the next two wash steps (with 500 μL of Buffer AW1, and 500 μL of Buffer AW2, respectively) were discarded. An appropriate amount of Buffer AE was added directly onto the DNeasy membrane, and the contents incubated at room temperature for at least 1 min, and centrifuged for 1 min at 6000 × *g* (8000 rpm) to collect the eluate. Extracted DNA can be stored at –20°C until used for PCR amplification and/or cloning.

PCR amplification and cloning

Extracted DNA (50–100 μg) from each sample was amplified by PCR. The quality of the DNA obtained from the formalin-fixed, paraffin-embedded samples was assessed by a parallel PCR amplification using primers for the β-globin gene, which serves as the 'housekeeping' or internal control gene—GlobinF primer (5′-ACA CAA CTG TGT TCA CTA GC-3') and the GlobinR primer (5′-CAA CTT CAT CCA CGT TCA CC-3'). Papillomavirus sequences were amplified by two complementary methods, both targeting highly conserved regions within the L1 open reading frame of the HPV genome. The first method, as mentioned previously, used the GP5+/GP6+ primers[6] and the second method used the FAP 59/64 primers.[7]

The first method utilises a HPV DNA genotyping chip (version 2.0; BiomedLab, Korea), a miniature microarray system to detect the PCR-amplified products. The genotyping chip contains 22 type specific probes that will identify 15 high risk HPVs (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, 68 and 69) and 7 low risk HPVs (6, 11, 34, 40, 42, 43 and 44). Any HPV DNA present in the extracted DNA samples would be amplified with the GP5d+ primer, 5'-TTTKTT ACHGTKGTDGATACYAC-3', and the GP6d+ primer, 5'GAAAHAT AAAYTGYAADTCAT AYTC-3' (the degeneracy at K, H, D, and Y represents G/T, T/A/C, A/T/G, and T/C, respectively). The amplified DNA products (of both HPV and β-globin) were then labelled with Cy5-dUTP (NEN; Life Science Products, USA), denatured by incubating with 3N sodium hydroxide solution (10% vol/vol) at room temperature for 5 min, neutralised by adding 1M Tris-HCl (pH 7.2, 5% vol/vol) and 3N hydrochloric acid (10% vol/vol), and finally cooled for 5 min on ice. A hybridisation solution made of 6 μL SSPE (saline sodium phosphate–EDTA buffer; Sigma Chemical Co, USA) and 0.2% sodium dodecyl sulfate, were added to the samples and applied to the DNA chip. Hybridisation was performed at 40°C for 2 h, followed by several washes (2 min each with 3 μL SSPE, and 1 μL SSPE). Any hybridised HPV DNA was left at room temperature (air-dried) and visualised by laser fluorescence scanning, using an Axon scanner. The expected size range of HPV amplicons is 140–150 bp.

The second method, as previously mentioned, involved the use of a pair of degenerate primers, FAP59/64, primarily aimed at the amplification of cutaneous HPV types. These primers were designed by Forslund *et al*.,[7] covering two regions manifesting a relatively high degree of nucleotide sequence identity in the 5' end of the L1 ORF. The positions of the primers FAP59 (5′-TAACWGTIGGICAYCCWTATT-3') and FAP64 (5'-CCWA-TATCWVHCATITCICCATC-3') corresponded to nucleotides 5981–6001 and 6458–6436 of the HPV-8 genome, yielding an amplicon of 478 bp. The FAP59 primer contained two inosine nucleotides and was degenerated at three positions, with a total degeneracy number of 8. The FAP64 primer also contained two inosines and was degenerated at four positions, with a total degeneracy number of 36. Degenerate nucleotides of primers: W = T,C; I = inosine; Y = C,T; D = A,G,T; B = G,C,T; H = A,C,T; V = A,C,G.

Briefly, extracted DNA was added to 0.4 μM each of the FAP59 and FAP64 primers, 20 μM of each deoxynucleotide triphosphate (dNTP; Boehringer Mannheim, Germany), 0.2% bovine serum albumin (BSA) and 1.0 U Taq DNA polymerase (AmpliTaq Gold; Roche, Switzerland) and reaction buffer (10X Gold Buffer). PCR was carried out in an automated thermocycler (ABI 9700; Applied Biosystems, USA), pre-programmed for the following PCR conditions: 10 min at 94°C and then 45 cycles of 1–5 min at 94°C, 1.5 min at 52°C and 1.5 min at 72°C.

Five μL of the amplified material was analysed by electrophoresis in 2% agarose gel (SeaKem; FMC Bioproducts, USA) in 1X TBE buffer[8] containing ethidium bromide (20 μL/mL; Boehringer Mannheim). The HPV-specific amplicons, if present, were identified by size determination in UV light. In cases with positive bands, for improving the quality of direct DNA sequencing, 5 μL of the amplicon was reamplified in 50 μL PCR mixture, and subsequently separated in 1.5% agarose gel (SeaKem) in 1X TAE buffer.[8] The expected amplicon size with the FAP primers is 478 bp. Note: Using this set of primers, HPV-40 (low risk mucinous HPV) and HPV-58 (high risk mucinous HPV) would give rise to bands of about 700 bp and 260 bp, respectively.

The amplicon (amplified PCR product) was then cut out from the gel and purified with a spin column (QIAquick PCR Purification Kit; Cat #28106; Qiagen), and eluted in 20 μL Buffer EB. Ten μL of sequencing reagent (ABI Prism, Dye Terminator Cycle Sequencing ready reaction kit FS; Applied Biosystems), comprised of 0.8 μL water, 4 μL BigDye Terminator, 0.2 μL primer (forward or reverse) and 5 μL of purified PCR product was used for the cycle sequencing reaction.

For the purification of the cycle sequencing reaction product, 10 μL of sterile double-distilled water, 2 μL of sodium acetate, pH 4.6 and 50 μL of 95% ethanol were added. The mixture was vortexed, placed on ice for 10 min, and centrifuged at 10 000 rpm for 15 min. The ethanol was aspirated off completely and replaced by 250 μL of 70% ethanol, followed by centrifugation for 15 min. The ethanol was again aspirated off and the pellet was air dried and kept in the dark. Just prior to its application to the sequencer, 15 μL of Hi-Di Formamide