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Investigating a cluster of vulvar cancers in young women: distribution of human papillomavirus and HPV-16 variants in vulvar dysplastic or neoplastic biopsies

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Abstract. ***Background*:** A high incidence of vulvar cancer, and its precursor lesion, high-grade vulvar intraepithelial neoplasia (VIN) has been identified in young Indigenous women living in the Arnhem Land region of the Northern Territory (NT) of Australia. This clustering is restricted to women aged <50 years, suggesting that oncogenic human papillomavirus (HPV) is a key causal factor. This study compared the HPV genotype prevalence, HPV-16 variant distribution and $p16^{INK4a}$expression in stored vulvar cancer and high-grade VIN biopsy specimens from women residing in Arnhem Land, with specimens taken from Indigenous and non-Indigenous women in other regions of NT where there is no observed increase in vulvar cancer incidence. ***Methods*:** Twenty high-grade VIN and 10 invasive cancer biopsies were assessed from Arnhem Land along with 24 high-grade VIN and 10 invasive cancer biopsies from other regions of NT. ***Results*:** Biopsies from Arnhem Land were similar to those from other regions in the detection of high-risk (HR) or possible HR HPV (VIN: 95% and 84% respectively for Arnhem Land and other regions, $P = 0.356$; invasive cancer: 100% and 80%, $P = 0.473$), HPV-16 (VIN: 60% and 80%, $P = 0.364$; invasive cancer: 70% and 70%, $P = 1.0$) and $p16^{INK4a}$ expression (VIN: 90% and 84%, $P = 0.673$; invasive cancer: 100% and 80%, $P = 0.474$). All HPV-16 variants were of the European prototype. ***Conclusion*:** Comparison of biopsies revealed no significant difference in the frequency of oncogenic HPVs or HPV-16 variant types between Arnhem Land and other regions, suggesting another cofactor in this cluster.

Additional keywords: Aboriginal, Australia, high-risk human papillomavirus, Indigenous, vulvar intraepithelial neoplasia.

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Background

Vulvar cancer is an uncommon gynaecological cancer,[1] largely delineated into two distinct groups.[2] Generally, in older women, the histological type is keratinising squamous cell carcinoma, which develops in areas of preceding squamous cell hyperplasia or is associated with vulvar dermatoses such as lichen sclerosus.[3] In younger women, the histology is that of warty basaloid squamous cell carcinoma and is associated with persistent infection with oncogenic human papillomaviruses (HPVs), particularly genotype 16.[4,5] Vulvar intraepithelial neoplasia (VIN) classified as high-grade (VIN 2 and 3) is considered to be the precursor lesion to HPV-related invasive vulvar cancer.[5] A large meta-analysis estimated that, among women across all age groups, HPV was present in 40.4% of invasive vulvar cancers and 85.3% of high-grade VINs,[6] with HPV-16 being the most frequently detected genotype.[4,6]

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Intratypic variability of HPVs exists in different parts of the world, with sequence variation within the *L1*, *E6* and *E7* genes of the HPV genome. For HPV-16, it has been suggested that some variants, namely the non-European variants, are generally associated with a greater risk of cervical neoplasia.[7] Polymorphisms in HPV-16 E6 T350G has also been found to be more prevalent in women with persistent infection and cervical neoplastic disease progression than the HPV-16 prototype 350T.[8] One study has examined the role of HPV variants in vulvar cancer and found that HPV-16 European variants were the most prevalent; however, this study was limited in that it was based on the examination of only nine vulvar specimens.[9]

During the past few decades, increases in incidence of high-grade VIN and vulvar cancer in younger women have been reported in several developed countries.[10–12] In Australia, clustering of VIN and vulvar cancer has been identified in younger Aboriginal (Indigenous) women residing in remote communities in the Northern Territory (NT) of Australia, particularly in the Arnhem Land region.[13] Between 1996 and 2005, the age-adjusted incidence of vulvar cancer in Indigenous women aged under 50 years in the eastern Arnhem Land district was 31.1 per 100 000, which is over 50 times higher than the national Australian rate for the same age group (0.4 per 100 000).[13] For Indigenous women living elsewhere in the Top End and Central Australia, incidence rates were 2.7 and 3.2 per 100 000, respectively.[13] Although the reason for this high incidence in Arnhem Land is unclear, the cases are restricted to Indigenous women aged less than 50 years, suggesting that infection with oncogenic HPV is a key causal factor.

This study aimed to explore the role of HPV in this cancer cluster by comparing vulvar biopsy specimens with specimens taken from Indigenous and non-Indigenous women in other regions of the NT where there is no clustering of cases for the detection of HPV genotypes, HPV-16 variants, E6 T350G and $p16^{INK4a}$ expression.

Materials and methods

The cluster of vulvar neoplastic disease in remote Indigenous communities in Arnhem Land has been described previously.[13] This study was approved by the Human Research Ethics Committee of the Menzies School of Health Research, Charles Darwin University and the NT Department of Health and Families with a waiver of individual patient consent. The study procedures conformed to the principles of the Declaration of Helsinki.

Sample selection

We identified 67 histologically diagnosed paraffin-embedded tissue biopsies taken from 59 women diagnosed with high-grade VIN or vulvar cancer collected between 1 January 1996 and 31 December 2005. Identification of biopsies and case definitions of high-grade VIN and invasive vulvar cancer have been reported previously.[13] Briefly, the process for identifying biopsies was from four data sources that were able to identify women residing in the NT with vulvar lesions: (a) the NT Cancer Registry; (b) the Colposcopy Database, maintained by Royal Darwin Hospital and Gynaecological Outreach Service (GOS), which contains details (including results) of all colposcopies performed by the GOS and public gynaecology services in the Top End since 1996; (c) an anogenital cancer histology results database of women with vulvar disease, maintained by the Royal Darwin Hospital Pathology Department; and (d) a separate database of women with vulvar disease.[13] The colposcopy database includes almost all colposcopies performed for Indigenous women in the Top End, with the exception of those provided by two private gynaecologists in Darwin, who treat a few Indigenous women and very few from remote communities.[13] Biopsy identification represents the total number of high-grade VIN and vulvar cancer biopsies diagnosed for the given timeframe in the NT.[13] Indigenous and non-Indigenous women from throughout the NT were included, with the aim of comparing HPV prevalence in biopsies from Arnhem Land women with all other biopsies from women living elsewhere in the NT. All 32 biopsies from Arnhem Land were from Indigenous women compared with 12 out of 24 (50%) high-grade VIN and 2 out of 11 (18%) vulvar cancer biopsies from elsewhere in the NT.

Women were identified as 'Indigenous' in this study based on the three standard Australian Indigenous identification criteria, namely Aboriginal and/or Torres Strait Islander descent, self-identification and community recognition. Indigenous status data was obtained primarily from the Client Master Index of NT Department of Health clinical information systems (which has been validated and found to have a very high level of accuracy),[14] supplemented by the treating clinicians' personal knowledge of the patients. Being considered Indigenous was not based on the geographical location of residency.

Tissue preparation and nucleic acid isolation

One section from each archival paraffin-embedded biopsy was analysed for HPV with the outer sections stained with haemotoxylin and eosin to confirm histological diagnosis.[15] To minimise cross-contamination, the microtome was cleaned with xylene after every sample was sectioned and a new microtome blade was used for each sample, with regular changing of gloves performed throughout. Sectioning of control lung tissue specimens was carried out randomly within the order of the specimens being sectioned.

Each section was de-paraffinised with 800 μL of histolene and subsequently washed with 400 μL of 100% ethanol and twice with 500 μL of 70% ethanol. The sample was air-dried, treated with 80 μL of Tissue Lysis Buffer (Roche Molecular Systems, Alameda, CA, USA) plus 20 μL of proteinase K, and placed on a 55°C heat block until fully digested. DNA was subsequently isolated on the automated Roche MagNa Pure LC using DNA Isolation Kit 1 (Roche Molecular Systems) and eluted in 100 μL of Elution Buffer (Roche Molecular Systems).

HPV genotyping

The INNO LiPA HPV genotyping test (Innogenetics, Ghent, Belgium) targets the amplification of a 65-bp amplicon of the L1 region of the HPV genome using reverse line blot hybridisation. This assay has the ability to simultaneously amplify and detect up to 28 different anogenital HPV genotypes (HPV-6, -11, -16, -18, -26, -31, -33, -35, -39, -40, -42, -44, -45, -51, -52, -53, -54, -56, -58, -59, -66, -68, -69, -70, -71, -73, -74 and- 82) with the inclusion of a 270-bp human DNA internal control, HLA-DPB1,

and two HPV controls, each control being a 65-bp region of the *L1* gene. Briefly, each reaction used 10 µL of DNA in a total polymerase chain reaction (PCR) volume of 50 µL and amplified as per the manufacturer's recommendations. Reactivity to probe lines was manually interpreted.[16]

HPV genotypes were classified into oncogenic groups according to the classification recommended by the International Agency for Research on Cancer:[17] high-risk (HR) genotypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58 and 59; probable HR genotype 68; possible HR genotypes 26, 53, 66, 73 and 82; and low-risk genotypes 6, 11, 40, 43, 44, 54 and 70. The risk category of genotypes 69, 71 and 74 is unknown.[18]

HPV-16 variant identification

HPV-16 positive DNA specimens were sequenced within *L1* and *E6* genes using the primer pair L1-F (5′-GTGTGACGC TATCAGCATGC-3′) and L1-R (5′-GTTCCTACTGCAATAC CGCAAG-3′), spanning a 169-bp region of the *L1* gene, and E6-F (5′-TGCAATGTTTCAGGACCCACA-3′) and E6-R (5′-CA GTAACTGTTGCTTGCATAC-3′), spanning across 118 bp of the *E6* region. HPV-16 *E6* genome variants in the T350G position were determined by using the primer pair E6-R (5′-TG CTGTTCTAATGTTGTTCC-3′) and E6-F (5′-GAATCCATA TGCTGTGAT-3′) spanning across 102 bp of the *E6* region (see Table 1). The PCR consisted of 10 µL of template DNA, 10× PCR Buffer (Applied Biosystems, Carlsbad, CA, USA), 2.5 mM $MgCl_2$ (Applied Biosystems), 0.2 mM of each Deoxynucleotide triphosphates (Bioline, London, UK), 5U/µL of TaqGold® DNA polymerase (Applied Biosystems) and 1 mM of each primer PCR in a 50 µL reaction volume. PCR products were visualised on a 2% agarose gel. PCR product purification was performed using Ampure (Agencourt Bioscience Corp., Beverly, MA, USA), as per manufacturer's instructions. DNA concentrations were estimated using a Qubit fluorometer (Invitrogen, Eugene, OR, USA).

High-throughput nucleotide Sanger sequencing of purified PCR products was performed at the Australian Genome Research Facility (Parkville, Vic., Australia) using an AB 3730×1 sequencer (Applied Biosystems). Both strands from each PCR product were sequenced in duplicate to ensure sufficient sequence overlap and fidelity.

p16^{INK4a} immunostaining

All biopsies were $p16^{INK4a}$ stained and a diffuse $p16^{INK4a}$ score was used as a marker for HPV *E7* transcription. Automated $p16^{INK4a}$ staining was performed using a DakoCytomation Autostainer (DakoCytomation, Glostrup, Denmark) and CINtec cytology kits (MTM Laboratories, Heidelberg, Germany) according to the manufacturers' instructions. Slides containing cytospun HeLa (10–50 copies HPV-18 per cell, HPV-positive) and human embryonic lung (HPV-negative) cell lines were utilised as immunostaining controls. Slides were graded on the basis of the percentage of the abnormal epithelium seen and according to the method previously published.[19] Briefly, $p16^{INK4a}$ stain was scored as negative if <1% of the abnormal cells displayed $p16^{INK4a}$ staining, sporadic positive if 1–5% of the cells were positive, focal positive if small cell clusters between 5% and 25% were positive and diffuse positive if >25% of the cells were positive.

Statistical analysis

Proportions, means and medians were calculated to summarise the data as appropriate.

The χ^2-test was used to compare biopsies from Arnhem Land women with biopsies from women elsewhere in the NT for the following outcomes: the proportion positive for any HR HPV (which included definite, probable and possible HR HPV genotypes), HPV-16, HPV-16 E6 G350T and $p16^{INK4a}$. To compare the average age of VIN and vulvar cancer cases between regions, an unpaired *t*-test was performed. Analyses were undertaken initially for all women and then restricted to Indigenous women only. Unless otherwise stated, *P*-values are given for the comparison between biopsies from Arnhem Land women and biopsies from all women (Indigenous and non-Indigenous) living in other parts of the NT. *P*-values less than 0.05 were considered statistically significant. All analyses were conducted using Stata ver, 10.0 (Stata Corporation, College Station, TX, USA).

Results

From the Arnhem Land region, two invasive vulvar cancer samples had negative internal controls or negative HPV genotypes as detected by the INNO LiPA genotyping test; these samples were excluded from further analysis. Overall, 20 high-grade VIN biopsies (from 17 women) and 10 invasive vulvar cancer biopsies (from nine women) were used for further analysis of cases from the Arnhem Land region.

From the other regions of the NT, one invasive vulvar cancer biopsy with a negative internal control or negative HPV genotypes as detected by INNO LiPA genotyping test was excluded from further analysis. Therefore, 24 high-grade VIN

Table 1. Sequence of primers, single nucleotide polymorphism position and length of amplicons generated to identify HPV-16 variants

Gene	Primer pair	Nucleotide change positions within region	Length of amplicon (bp)
L1	5′-GTGTGACGCTATCAGCATGC-3′ 5′-GTTCCTACTGCAATACCGCAAG-3′	6695 : 6721 : 6803	169
E6	5′-TGCAATGTTTCAGGACCCACA-3′ 5′-CAGTAACTGTTGCTTGCATAC-3′	131 : 132 : 143 : 145 : 178	118
E6 T350G	5′-TGCTGTTCTAATGTTGTTCC-3′ 5′-GAATCCATATGCTGTGAT-3′	350	102

(from 24 women) and 10 invasive vulvar cancer biopsies (from nine women) from other regions of the NT were available for analysis.

Of the total assessable biopsies (64) with adequate internal control results (from 59 women), 20 (31%) were from non-Indigenous women and 44 (69%) from Indigenous women. All biopsies ($n = 30$) from the Arnhem Land region were from Indigenous women, whereas 14 out of 34 (41%) from the surrounding areas were from Indigenous women ($P < 0.001$). Women from Arnhem Land were younger than those from other regions for all women combined (mean age: 35.2 years, s.d. 8.5 years *v.* 46.4 years, s.d. 15.2 years; $P = 0.0007$) and for Indigenous women only (35.2 years, s.d. 8.5 years *v.* 41.1 years, s.d. 10.3 years; $P = 0.05$).

Overall, 40 out of 44 (91%) high grade VIN and 17 out of 20 (85%) vulvar cancer biopsies were positive for HPV. A further 6 out of 64 biopsies (four high-grade VIN – one Indigenous woman and three non-Indigenous women– and two vulvar cancers, both from non-Indigenous women) (9%) were found to be negative for any HPV genotype on the INNO LiPA test, including one biopsy from Arnhem Land. One high-grade VIN biopsy from Arnhem Land (histologically classified as high-grade VIN) was found to be human DNA internal control positive and HPV controls 1 and 2 positive (on the INNO LiPA test). Further, this biopsy was positive for sporadic $p16^{INK4a}$ staining and negative for HPV by the INNO LiPA HPV genotyping test. Further, this biopsy was positive for $p16^{INK4a}$ sporadic staining; however, it was negative for any HPV genotype on the INNO LiPA HPV genotyping test. This sample was counted as HPV-positive, genotype unknown, and is included in Table 2. This sample was retested to confirm this result, which was again positive for the human DNA internal control and HPV controls 1 and 2.

The distribution of HPV genotypes in high-grade VIN biopsies is shown in Table 2. In biopsies from Arnhem Land women, HPV was detected in 20 out of 20 (100%), with HPV-16 being the most frequently detected single HPV genotype (11 out of 20, 55%). In biopsies from women in other regions of the NT, HPV was detected in 20 out of 24 (84%) biopsies, with HPV-16 detected in all single infections. Comparison of high-grade VIN biopsies for the two regions revealed no significant difference in the detection of any HR HPV (including possible and probable HPV) ($P = 0.356$) or detection of HPV-16 ($P = 0.364$). There was no significant difference in HR HPV or HPV-16 prevalence between women from the two regions (Arnhem and other) when analyses were restricted to Indigenous biopsies only; however, this comparison was limited due to small sample numbers ($n = 12$). Histologically, one biopsy from a non-Indigenous woman residing in the 'other regions' was classified as differentiated VIN (4%).

The distribution of HPV genotypes in vulvar cancer biopsies is shown in Table 3. In biopsies from Arnhem Land women, HPV was detected in 10 out of 10 (100%), with HPV-16 being the most frequently detected (7 out of 10, 70%). In biopsies from women in other regions of the NT, HR and possible HR HPV genotypes were detected in 8 out of 10 (80%), with HPV-16 detected in six out of seven (86%) single infections. Comparison of the two regions revealed no significant difference in the detection of any HR HPV ($P = 0.474$) or the detection of HPV-16 ($P = 1.0$). Analyses restricted to Indigenous women

Table 2. Detection of HPV genotypes in single and multiple infections in Arnhem Land region compared with other regions for high-grade vaginal intraepithelial neoplasia (VIN) biopsies

	Arnhem Land		Other regions[A]			
	All Indigenous		Indigenous		Non-Indigenous	
	$n = 20$	%	$n = 12$	%	$n = 12$	%
HPV-positive	20	100.0	8	66.7	7	58.3
HPV-positive or genotype unknown	1	5.0	–	–	–	–
HPV single infections						
HPV-16	11[C, D, E]	55.0	8	66.7	7	58.3
HPV-33[B]	1[E]	5.0	–	–	–	–
HPV-39	1[G]	5.0	–	–	–	–
HPV-26	2	10.0	–	–	–	–
HPV-53	1	5.0	–	–	–	–
Multiple infections						
	11 and 35	5.0	16 and 59	8.3	16 and 56[F]	8.3
	66 and 82	5.0	16 and 52	16.7	51 and 82	8.3
	16 and 33[B,G]	5.0				
HPV-negative	–	–	1	8.3	3	25.0
$P16^{INK4a}$ positive (any)	18	90.0	11	91.6	9	75.0

[A]Includes all health district regions of the NT except the Arnhem Land region.
[B]Coinfection with HPV-52 and HPV-54 could not be ruled out.
[C]For one sample, coinfection with HPV-74 could not be ruled out.
[D]Includes two biopsies from the same woman.
[E]Includes one biopsy from each HPV genotyping result from the same woman.
[F]One biopsy was histologically classified as differentiated VIN.
[G]Includes one biopsy from each HPV genotyping result from the same woman.

Table 3. Detection of HPV genotypes in single and multiple infections in Arnhem Land region compared with other regions for invasive vulvar squamous cell carcinoma biopsies

	Arnhem Land		Other regions[A]			
	All Indigenous		Indigenous		Non-Indigenous	
	n = 10	%	*n* = 2	%	*n* = 8	%
HPV-positive	10	100.0	2	100.0	6	62.5
High-risk HPV single infections						
HPV-16	7[C]	70.0	2	100.0	4[D]	50.0
HPV-33[B]	1	10.0	–	–	1	12.5
HPV-35	1	10.0	–	–	–	–
HPV-52	1	10.0	–	–	–	–
Multiple infections						
	–	–	–	–	16 and 66	12.5
HPV-negative	–	–	–	–	2[E]	25.0
P16^{INK4a} positive (any)	10	100.0	2	100.0	6	75.0

[A]Includes all health district regions of the NT except the Arnhem Land region.
[B]Coinfection with HPV-52 and HPV-54 could not be ruled out.
[C]Includes two biopsies from the same woman.
[D]Two biopsies were histoloigcally diagnosed as differentiated vulvar cancer; these two biopsies are from the same woman.
[E]Two biopsies were histologically classified as differentiated vulvar cancer.

only were not carried out due to small numbers. Histologically, four vulvar cancer biopsies from non-Indigenous women residing in the 'other regions' were classified as differentiated vulvar cancer (40%).

Sensitivity analysis was performed by combining high-grade VIN and invasive vulvar cancer biopsies. For the detection of HR HPV and HPV-16, there were no statistically significant differences detected between the two regions, ($P = 0.109$ and $P = 0.284$ respectively).

All HPV-16 positive biopsies from women in the two regions were found to be of the European variant of HPV-16. For the three single nucleotide polymorphisms (SNPs) contained in the *L1* gene, all HPV-16 positive biopsies were (A : G : A), similar to the HPV 16 European prototype. For the five SNPs contained in the *E6* gene, all HPV-16 positive biopsies were (A : G : C : G : T), similar to the HPV-16 European prototype; GenBank ID AF536179.1) A comparison of Arnhem Land to the other regions for the T350G variant on the HPV-16 *E6* gene is shown in Table 4. The 350G variant was more commonly detected in biopsies from other regions with seven (37%) and two (29%) detected in high-grade VIN and squamous cell carcinoma biopsies respectively. However, comparison of the two regions revealed no statistically significant difference in the detection of the HPV-16 *E6* 350 variant in high-grade VIN or invasive vulvar cancer biopsies ($P = 0.108$ and $P = 1.0$, respectively).

Of the high-grade VIN biopsies from Arnhem Land women, expression of p16^{INK4a} was detected in 18 out of 20 (90%) biopsies, compared with 11 out of 12 (92%) Indigenous and 9 out of 12 (75%) non-Indigenous biopsies from other regions ($P = 0.673$). For invasive vulvar cancer biopsies, all 10 biopsies from women residing in Arnhem Land had p16^{INK4a} expression, as did the two (100%) Indigenous biopsies and six out of eight (75%) non-Indigenous biopsies from other regions ($P = 0.474$).

Discussion

The incidence of high-grade VIN and vulvar cancer in younger Indigenous women residing in the Arnhem Land region is, to our knowledge, the highest reported worldwide.[11,13] This study found that there was no significant difference in the prevalence of HR HPV or the type-specific distribution of HPV in high-grade VIN or vulvar cancer biopsies from women in Arnhem Land compared with biopsies from women in other regions in the NT where there is little, if any, excess of vulvar neoplasia.

HPV-16 was the most frequently detected genotype in both regions (Arnhem Land and other parts of the NT). This is consistent with data from several meta-analyses of HPV genotype prevalence in high-grade VIN as well as vulvar

Table 4. Comparison of detection of the base change at the HPV-16 E6 350 position (T → G) for high-grade vulvar intraepithelial neoplasia and invasive squamous cell carcinomas

	Arnhem Land region		Other regions[A]				*P*-value[B]
			Indigenous		Non-Indigenous		
	n (*N*)	%	*n* (*N*)	%	*n* (*N*)	%	
High-grade VIN	1 (12)	8.3	4 (11)	36.4	3 (8)	37.5	0.108
Squamous cell carcinoma	1 (7)	14.3	0 (2)	0	2 (5)	40.0	1.0

[A]Includes all health district regions of the NT except the Arnhem Land region.
[B]*P*-values are for comparisons between biopsies from Arnhem Land women with biopsies from all women (Indigenous and non-Indigenous) living in other regions of the NT.

cancer,[4,6] plus individual studies of invasive vulvar cancer biopsies in younger women in other countries.[20–22] HPV-18 was not detected in any biopsies in this study[21,22] and this may be due to a difference between HPV genotypes and tropism for vulvar epithelia.

This is the first study to observe HPV genotype, variant type and $p16^{INK4a}$ expression in the same cohort of samples, and the largest study investigating HPV variant type in vulvar dysplastic and neoplastic biopsies. One small study of vulvar cancer biopsies ($n = 9$) from United States women showed HPV-16 European variants were the most prevalent, and this correlated with the patients' ethnic background.[9] In our study, sequence data for the *L1* and *E6* regions of the HPV-16 positive VIN and squamous cell carcinomas showed similar variations to the European prototype and there was no significant difference in sequence variation observed between the two regions.

Previous studies in cervical cancer have suggested that the variation in the HPV-16 E6 T350G may increase the risk of persistent infection.[23] This is the first study examining the presence of such variants in VIN and vulvar cancer biopsies; however we found no significant difference in the distribution of E6 T350G variants between Arnhem Land and other regions. This suggests that the very high incidence of vulvar cancer and high-grade VIN in Arnhem Land is unlikely to be due to a more virulent strain of HPV-16 in circulation. In addition, there was no observed difference in the extent of expression of $p16^{INK4a}$, as an indirect marker of HPV E7 transcription, between Arnhem Land and other regions. This is consistent with other studies of VIN and vulvar cancer,[24–26] suggesting there is no difference in the transcriptional activity of HPV infection in this population.

In our study, six specimens were found to be negative for detection of HPV. Upon histological review of these biospies (from sections either side of the PCR-tested sections), all contained reports of koilocytosis, who are pathoformic of HPV infection although they are not pathognomonic of HPV infection. HPV negativity could possibly be due to DNA fragmentation[27] as $p16^{INK4a}$ expression was observed in two of these samples and all samples were human DNA internal control positive. Alternatively, material from the remaining specimens was considerably smaller, which may have resulted in fewer copy numbers of HPV to detect. In addition, although no statistical differences were observed between the two studied regions, the study was limited by the relatively small sample size, reducing the statistical power to detect small statistical differences between regions. Therefore caution must be noted when examining these results.

This study is part of a long-term investigation into the causes of the high incidence of VIN and vulvar cancer in the Arnhem Land region. These findings do not support the epidemiology of this cluster as being due to a significant difference in the frequency of oncogenic HPV or a more virulent type of oncogenic HPV. We have observed a significant difference in age of diagnosis in this study, which may be due to environmental agents or inherited susceptibility to the oncogenic effects of HPV, and these factors are currently being investigated. Further, results from the previous epidemiological study, (confirming the clustering of high-grade VIN and vulvar cancer cases in the Arnhem Land region) demonstrate that 49% of Indigenous women in the NT had previously or subsequently been diagnosed with neoplastic or preneoplastic lesions of the cervix, vagina or anus,[13] with cervical lesions being the most common diagnoses (42%).[13] For women residing in Arnhem Land, 58% had previous or subsequent diagnoses with some other anogenital disease, with cervical lesions being the most common diagnoses (50%).[13] However, for those aged 0–49 years, cervical cancer incidence rates are similar for Arnhem Land women, Indigenous women living elsewhere in the NT and non-Indigenous women living in the NT.[13] Therefore other possibilities such as genetic or environmental factors, or both, may be the cause. Cofactors such as smoking and immunosuppression may increase the risk of vulvar neoplasia.[28,29] Smoking is more common amongst Indigenous women in the NT than in other Australian women; however, the proportion of Indigenous female smokers in the Arnhem Land area is similar to that present in other areas of the NT.[30] The prevalence of HIV infection in the Arnhem Land region is unknown; however, the proportion of HIV notifications is consistently lower in Indigenous than non-Indigenous people in the NT.[31] A higher prevalence of cofactors such as smoking and HIV infection are also unlikely to completely explain the very high disease incidence in this population.

It is also possible that there are heritable genetic risk factors in this population, which may impair host immunity to HPV. Although there has been limited investigation of genetic susceptibility to vulvar cancer, there is some support for an immunogenetic contribution, with evidence of polymorphisms in genes encoding human leukocyte antigen,[32] and an interaction between genetic variation in Th1 cytokines and smoking in vulvar neoplasia.[28]

This study found no evidence that a more virulent strain of HPV-16 was related to the very high incidence of VIN and vulvar cancer in young Indigenous women living in the Arnhem Land region. Alternative explanations for this cancer cluster, such as an environmental agents or inherited susceptibility to oncogenic effects of HPV that is localised to the vulva or topical application of a carcinogen, are currently under examination.

Conflicts of interest

SMG has received advisory board fees and grant support from CSL Biotherapies (CSL) and GlaxoSmithKline (GSK); lecture fees from Merck, GSK and Sanofi Pasteur; funding (through her employing institution) to conduct HPV vaccine studies for Merck, Sharp and Dohme (MSD) and GSK; and is a member of the Merck Global Advisory Board and the Merck Scientific Advisory Committee for HPV. This relates to work outside of this submitted manuscript. SET is the recipient of a GSK Australian Postgraduate Support Grant for work outside of this submitted manuscript. CSL have provided funding to the authorship group to support a workshop on genetic susceptibility to vulvar cancer. There was no funding for this study beyond that stated below from the National Health and Medical Research Council.

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