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Runs of homozygosity and a cluster of vulvar cancer in young Australian Aboriginal women

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HIGHLIGHTS

- We performed a genetic study of a vulvar cancer cluster in Arnhem Land women.
- No effects of genome-wide homozygosity or individual ROHs were observed.
- Prior diagnosis of CIN was associated with diagnosis of vulvar cancer or VIN.

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ABSTRACT

Objective. A cluster of vulvar cancer exists in young Aboriginal women living in remote communities in Arnhem Land, Australia. A genetic case–control study was undertaken involving 30 cases of invasive vulvar cancer and its precursor lesion, high-grade vulvar intraepithelial neoplasia (VIN), and 61 controls, matched for age and community of residence. It was hypothesized that this small, isolated population may exhibit increased autozygosity, implicating recessive effects as a possible mechanism for increased susceptibility to vulvar cancer.

Methods. Genotyping data from saliva samples were used to identify runs of homozygosity (ROH) in order to calculate estimates of genome-wide homozygosity.

Results. No evidence of an effect of genome-wide homozygosity on vulvar cancer and VIN in East Arnhem women was found, nor was any individual ROH found to be significantly associated with case status. This study found further evidence supporting an association between previous diagnosis of CIN and diagnosis of vulvar cancer or VIN, but found no association with any other medical history variable.

Conclusions. These findings do not eliminate the possibility of genetic risk factors being involved in this cancer cluster, but rather suggest that alternative analytical strategies and genetic models should be explored.

Introduction

Cancer of the vulvar is relatively rare, and usually occurs in postmenopausal women [1]. However, the incidence of vulvar cancer among Indigenous women aged less than 50 years living in remote communities in the East Arnhem district of the Northern Territory of Australia (see Fig. S1) is more than 70 times the national incidence rate in the same age group (31.1 per 100 000 compared with 0.4 per 100 000) [2]. Vulvar cancer in pre-menopausal women, as found in communities within the East Arnhem district and several communities bordering that district (hereafter referred to as Arnhem Land), is associated with persistent human papillomavirus (HPV) infection, particularly genotype 16, whereas in older women it is more usually associated with a dermatological condition called lichen sclerosus [3–5]. The precursor lesion to HPV-related invasive vulvar cancer is vulvar intraepithelial neoplasia (VIN) usual type (warty, basaloid and mixed) [6], and the incidence of VIN in this population is similarly high (34.7 per 100 000 in women aged less than 50 years) [2].

Previous work with the Arnhem Land population, however, found no evidence that higher rates of HPV infection [7] or that particularly virulent strains of HPV [8] could explain the excess incidence of vulvar cancer in this population, suggesting the possible involvement of additional environmental and/or genetic factors that may impair host immunity. Several environmental factors, including smoking and some sexually transmissible infections (such as gonorrhea and herpes simplex virus 2), have previously been found to be associated with

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increased risk of vulvar cancer [9–12]. However, these factors are unlikely to account for the cluster in Arnhem Land, as they occur at similar rates to that found in other Indigenous populations in the Northern Territory where there is no excess of vulvar cancer [13,14]. Of greater possibility is a hereditary cofactor, based on evidence of familial clustering of HPV-associated cancers observed in the Swedish population [15], combined with reports from gynecologists servicing the Arnhem district that the cases appear to occur in distinct family groups.

To date, little work has been undertaken investigating genetic risk factors involved in vulvar cancer, partly as a result of its relative rarity and the consequent difficulty in achieving an adequate sample size to detect real effects. A recent population based case–control study in the United States used a candidate gene approach to identify a suggestive association between common variants within the TNF gene region and increased risk of vulvar cancer [16]. If the cancer cluster in Arnhem Land has a genetic basis, however, the variant(s) involved is likely to be rare, to have a large effect size, and to stem from a mutation in a common ancestor. The affected communities in Arnhem Land comprise predominantly Indigenous populations, ranging in size from several hundred to approximately 2500 people. The small populations and extremely remote locations of the affected communities suggest the potential involvement of recessive effects resulting from increased parental relatedness, as disease alleles are more likely to be inherited identical-by-descent, although alternative population genetic scenarios are possible.

This mechanism could act either through recessive variants of large effect sizes, or through the cumulative influence of genome-wide homozygosity linking many recessive variants of small effect size [17]. Genetic association studies in small, isolated populations can be limited by power considerations, and by the presence of extended regions of homozygosity. However, analytical approaches utilizing these homozygous regions present a useful, and hitherto relatively neglected, complementary approach to allelic, genotypic and haplotypic association studies [18]. The possibility that the etiologies of complex diseases include recessive alleles at many genetic loci, each of small individual effect, and that increases in genome-wide homozygosity resulting from consanguineous pairings could increase the risk of these diseases, was first proposed a decade ago [19]. Since that time, a number of studies have assessed the influence of homozygosity in various diseases by employing high-density single nucleotide polymorphism (SNP) data to estimate the proportion of the autosome in runs of homozygosity (ROHs), resulting from inheriting identical haplotypes from each parent.

These ROHs are more common in outbred populations than previously thought, and their average length is proportionate to the number of generations since the common ancestor; longer runs (>5 Mb) are associated with recent parental relatedness, and shorter runs are indicative of ancient parental relatedness [17]. Associations have been identified between whole-genome ROH burden and schizophrenia [20] and with intellectual disability in simplex autism [21], although other studies found no association with survival to old age [22], risk of breast and prostate cancer [23], colorectal cancer [24], or multiple sclerosis [25]. More recently, other studies have extended the ROH analysis to identify specific loci associated with disease. This homozygosity mapping approach has been successful in identifying candidate loci for rheumatoid arthritis [26], human adult height [27], schizophrenia [28], autism spectrum disease [29] and Alzheimer Disease [30,31].

Because these analytical approaches are relatively new and consensus regarding defining criteria are yet to be firmly established, these studies employed a range of thresholds for defining ROHs [32]. Howrigan and colleagues [33] went some way towards addressing this problem by using simulation to assess the power of various methods of detecting autozygosity; importantly, their work confirmed the need to prune datasets for linkage disequilibrium (LD) and provided recommendations for thresholds aimed at detecting ancient and recent autozygosity. Accurately detecting regions inherited identical-by-descent increases the likelihood of identifying rare, semi-recessive genetic variants involved in disease etiology.

The current study aims to assess the role of partially recessive, deleterious variants in the vulvar cancer cluster in young Aboriginal women in Arnhem Land by investigating autozygosity assessed by genome-wide ROH burden and single ROH association mapping. A secondary aim is to investigate possible environmental cofactors using data extracted from participants' clinical records, relating to smoking and prior diagnoses of infections and HPV-related neoplasia.

Materials and methods

Study population

A genetic case–control study of the vulvar cancer cluster among young Aboriginal women resident in Arnhem Land was undertaken in 2011–2013, in seven Indigenous communities and a number of smaller outstations identified based on our earlier work [2,7]. These sites represent the majority of the affected communities. Given the sensitive nature of the research, prior to commencing the study, the research team consulted with elders, health boards and health services in the affected communities about the nature of research, the most appropriate ways to proceed as well as appropriate research dissemination strategies, as described previously [34]. Furthermore, an Indigenous Reference Group (IRG) that had been established during our previous study was reconstituted to advise on all aspects of the study. Ethical approval was received from the Top End Human Research Ethics Committee in 2011.

Using data from the Northern Territory Cancer Registry and the Gynaecology Outreach Service colposcopy database, women were identified who met the following criteria: had been diagnosed with vulvar cancer or high-grade VIN between 1996 and 2011; identified as Aboriginal and/or Torres Strait Islander; and their usual place of residence was in a community in Arnhem Land. Of the 34 women who met these criteria and were living, 30 were recruited to the study, as well as 62 unaffected controls, matched for age and community of residence. All participants gave written informed consent and provided two saliva samples using Oragene (OG-500) DNA collection tubes (DNAGenotek, Ottowa, Ontario, Canada). Participants were asked about their smoking status (at the time of diagnosis for cases, and at the time of recruitment for controls). Medical records from community primary health centers were used to confirm participants' date of birth, and their case/control status from previous Well Women's Screening health checks (an assessment which includes a Pap smear, vulvar examination, and discussion of sexual and reproductive health issues). Medical records were also accessed to extract information about any concurrent or previous diagnosis of cervical intraepithelial neoplasia (CIN) and any concurrent or previous infection with syphilis, gonorrhea, trichomoniasis, chlamydia or other sexually transmissible infections (STI). In instances where a control had not recently participated in a Well Women's Screening health check, they were offered screening in conjunction with community primary health centers and the NT Centre for Disease Control Sexual Health coordinator.

Global comparative data for genomic ROH in different populations were sourced from the Human Genome Diversity Project (HGDP) [35], adapting the methods used by Kirin and colleagues [36]. The HGDP has a controversial history with Australian Aboriginal populations [37]. As no suitable alternative could be found, use of this data was only undertaken after consultation with the IRG, who approved its use for comparative purposes.

Genotyping

Genomic DNA was extracted from saliva samples, according to the manufacturer's directions, and DNA concentration and purity were assessed using a NanoDrop 8000 (Thermo-Fisher Scientific) and

PicoGreen fluorometry. Samples were genotyped using Illumina HumanOmni 2.5 BeadChips (Illumina, San Diego, CA, USA). One control participant's sample showed evidence of contamination, and was removed, leaving 30 cases and 61 controls. Of the 2 379 855 SNPs genotyped, 59% were monomorphic. SNPs with a minor allele frequency of less than 0.05 were removed (n = 1 406 951), as were SNPs with a call rate of less than 90% (n = 8727). No individuals were removed for low genotyping (<99%). SNPs which deviated from the Hardy–Weinberg equilibrium ($p < 0.000001$) were excluded from the analysis (n = 377) and non-autosomal SNPs (n = 25 557) were also removed, resulting in a total of 941 976 SNPs included in the analysis.

To account for possible underlying population structure and to identify outliers, the dataset was pruned for LD and then analyzed using EIGENSOFT principal components analysis. No significant outliers were detected, but the first two principal components were identified as important to include in subsequent analyses to adjust for ancestry differences. Results were discussed with the IRG and other community members to determine the consistency of analyses with local understanding of relatedness between communities.

The HGDP dataset includes 1043 individuals from 51 populations, genotyped using the Illumina 650Y array [35]. For the global comparative analysis, autosomal SNPs present in both the Arnhem Land and HGDP datasets were extracted and merged (n = 241 139), and filtered for QC. No individuals were removed for low genotyping (<95%), 85 SNPs were removed for low call rate (<90%), 1519 SNPs were removed for low frequency (MAF < 0.05) and no SNPs significantly deviated from the Hardy–Weinberg equilibrium ($p < 0.000001$), leaving a total of 239 636 SNPs.

Runs of homozygosity (ROH)

Data were pruned for LD in PLINK v.1.07 [38], using a sliding window 50 SNPs in length, moving the window 5 SNPs each time, and with a variance inflation factor threshold of 2 [33]. ROHs were identified using the 'Runs of Homozygosity' program in PLINK, employing the thresholds summarized in Table 1. These thresholds were determined based upon Howrigan and colleagues' [33] recommendations for optimal autozygosity detection, adapted to suit the data, and to facilitate comparison with the global data. After pruning, 115 278 SNPs were retained. The length of the genotyped autosome was 2720.177 Mb. SNP coverage density was 23.6 kb/SNP, indicating that the minimum number of SNPs in a ROH would be 21, given a minimum length of 500 kb. However, as the density of SNPs is not consistent over the genome, minimum SNPs were set at 15 per 500 kb to ensure identified runs have sufficient SNPs to constitute a genuine ROH. One heterozygous call per window was allowed to account for genotyping error.

Similarly, the merged Arnhem Land and HGDP dataset was pruned for LD (leaving 99 166 SNPs) and ROHs were identified, using the thresholds in Table S1.

F_{roh} estimates

Two measures of genome-wide homozygosity were produced in PLINK. Observed homozygosity (H_{obs}) is simply the percentage of SNPs that are homozygous. F_{roh}, on the other hand, provides a genomic measure of autozygosity by calculating the proportion of the genotyped autosome in ROHs, for varying ROH lengths [17,25].

Statistical analysis

SNPs appearing in both the Arnhem Land and HapMap datasets were identified, and then a subset of these that were not in LD in the Arnhem Land participants was extracted. These markers were used to produce multidimensional scaling plots in PLINK and R v.2.15 [39].

Using the GenABEL package in R, the measures of genome-wide homozygosity were included in hierarchical general linear model estimations of a polygenic model, adjusted for the two identified principal components where necessary [40]. The polygenic model further accounted for the violation of the assumption of independent observations, introduced by cryptic relatedness within the sample, by including a matrix of pair-wise relatedness based on identity-by-state (IBS), weighted for allele frequency. The same methods were used to examine the effect of smoking, STIs (aggregated and by specific infection type) and history of CIN. A logistic regression model using case/control status as the outcome variable was used for all analyses.

Pools of overlapping runs, with corresponding consensus regions, were created in PLINK using the *--homozyg-group* command. The effect of consensus regions on case/control status was tested using Fisher's exact tests, with adjustments for relatedness to be made on any regions found to be significant. The mean length of the genome in ROHs of different sizes in the Arnhem Land sample was compared with global populations categorized by continent, using Fisher's exact tests, with significantly associated regions to be adjusted for relatedness. The mean length of the genome in long or shorter ROHs in the Arnhem Land participants was compared with global populations categorized by continent.

Table 1
Summary of the characteristics of the cases and controls.

Characteristic	Cases (%)	Controls (%)
Number	30	61
Age (years)		
Age at diagnosis		
Mean	37.63	
Range	25–62	
Age at recruitment		
Mean	44.07	38.34
Range	26–65	22–71
Diagnosis		
Invasive vulvar cancer	15 (50%)	
Vulvar intraepithelial neoplasia	15 (50%)	
Medical history		
Smoking[a]	24 (80%)	41 (67%)
Syphilis	11 (37%)	11 (18%)
Gonorrhea	6 (20%)	11 (18%)
Trichomoniasis	17 (57%)	24 (39%)
Chlamydia	6 (20%)	15 (25%)
Other STI	5 (17%)	4 (7%)
Any STI	23 (77%)	42 (69%)
Cervical intraepithelial neoplasia	22 (73%)	14 (23%)

[a] Defined as current smoker at the time of diagnosis among cases and at the time of recruitment for controls.

Results

Descriptive statistics

Participant characteristics, including those extracted from clinical records, are summarized in Table 1.

Principal components analysis

A principal components analysis of the Arnhem Land sample (see Fig. 1) displays a level of population stratification that is congruent with local understanding of relatedness between these communities. Notably, the pink community and the dark blue community are linguistically and culturally distinct from each of the other communities, and this is reflected in the genetic data. The apparent skew of the red community is the result of a cluster of closely related cases.

A summary of genetic variation between the Arnhem Land and HapMap populations demonstrates that the Arnhem Land population is relatively homogeneous when analyzed within a global context, despite the stratification identified above (see Fig. S2). Although the positioning of the Arnhem Land outliers appears to suggest that greatest

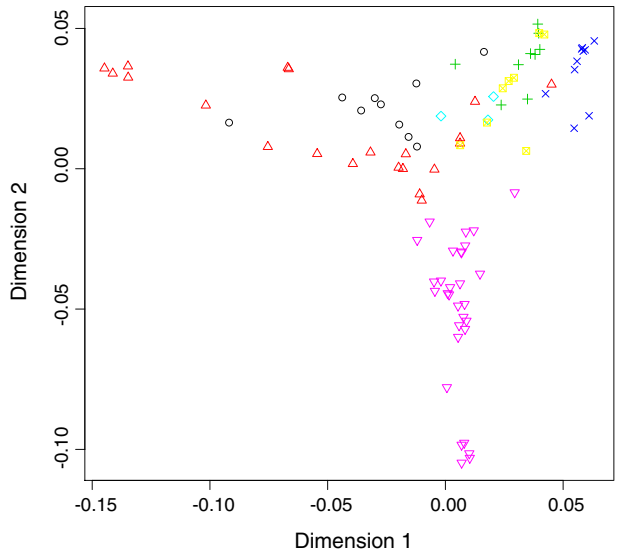

Fig. 1. Multidimensional scaling analysis of the Arnhem Land sample. Community of residence is indicated by color, as the IRG requested that individual communities not be identified by name.

similarity is with people of Mexican Ancestry in Los Angeles, comparison with the third and fourth dimensions (see Figs. S3 and S4) reveals this to be an artifact, resulting from recent admixture with Australians with ancestry from northern and western Europe.

Genome-wide ROH burden

No effect of genome-wide homozygosity was found on vulvar cancer or VIN case/control status for any measure of genome-wide homozygosity (see Table 2). Similarly, no evidence was found for an effect of smoking or STI (aggregated or specific infection). Previous history of CIN diagnosis, however, was found to be significantly associated with case status (see Table 3).

To place this sample within a global context, the distribution of long and shorter runs in the Arnhem Land sample was compared to the 51 HGDP populations, categorized by continent (see Fig. 2) [35,36]. For both the mean sum of ROH longer than 0.5 Mb, and the mean sum of long ROH (>5 Mb), the Arnhem Land participants were close to the global average.

Single ROH association mapping

Consensus regions, for which two or more individuals shared a homozygous region, were identified for ROH greater than 0.5 Mb in length. The number of individuals sharing a consensus region ranged from 2 to 23, and there were 1141 consensus pools identified. These were tested for association with case/control status, but none were significant after adjustment for multiple testing (see Table 4). The top consensus region had 6 cases and no controls sharing. This corresponded to a p-value of 0.0009, much larger than the Bonferroni-corrected threshold for significance of 4.4×10^{-5} (based on 1141 multiple tests). If there was a consensus region that was only found in 9 cases, genome-wide significance would be observed, suggesting that there is sufficient power to detect a homozygous disease-causing variant that explains a third or more of cases.

Table 2
Regression coefficients for the association between measures of genome-wide homozygosity and vulvar cancer case/control status.

Measure of homozygosity	β coefficient	p-value
H_{obs}	0.05	0.31
F_{roh} >0.5	2.37	0.35
F_{roh} >5	4.94	0.29

Table 3
Regression coefficients for the association between medical history and vulvar cancer case/control status (* significant at $p < 0.05$).

Medical history	β coefficient	p-value
Smoking	0.19	0.32
Syphilis	0.22	0.18
Gonorrhea	0.07	0.66
Trichomoniasis	0.11	0.46
Chlamydia	−0.03	0.81
Any STI	0.04	0.78
CIN	0.42	0.015*

Discussion

This investigation found no evidence of an effect of genome-wide homozygosity on vulvar cancer and VIN in Arnhem Land Aboriginal women. Although the consistently positive β coefficients in Table 2 seem to suggest that an increase in homozygosity is correlated with case status, these tests are not independent, and this observation is an artifact. Furthermore, no individual ROH was found to be associated with diagnosis of vulvar cancer and VIN. These results provide no evidence for the involvement of autozygosity, either through whole-genome ROH burden or the effect of a single ROH.

While the Arnhem Land sample size is relatively small, limiting the power to detect real effects, the fact that the incidence of vulvar cancer among young Indigenous women in this district is more than 70 times the national incidence rate suggests that a genetic risk factor (either individually or cumulatively across the genome), if involved, would be of sufficient effect size to be detectable in this sample.

Interestingly for a small and isolated population, no evidence was found that the Arnhem Land participants have more or longer ROHs than expected. This finding is perhaps a reflection of the effectiveness of the traditional kinship system in avoiding the effects of consanguineous pairings. For the Aboriginal peoples of Arnhem Land, kinship is a complex system that forms a core component of their identities and their understanding of the world and which dictates, among many other things, appropriate marriage partners [41].

It is noteworthy that the mean total ROH lengths for the HGDP dataset calculated for the present study are substantially smaller than those published previously by Kirin and colleagues [36]. This result is attributable to the fact that the previous study did not prune for LD before identifying ROH. Pruning for LD and low MAF SNPs was subsequently shown by Howrigan and colleagues [33] to be important in avoiding identifying chance (non-autozygous) ROH.

Although F_{roh} has been most commonly used in recent studies, it is by no means the only method for estimating inbreeding. Traditionally, estimates of F were based upon pedigree data, but the utility of this approach is limited by practical difficulties associated with collecting and verifying pedigree data, particularly when examining more ancient parental relatedness. These difficulties are magnified when working with remote Indigenous communities, with incomplete or non-existent written records, cultural and linguistic barriers, and different concepts of kinship, which encompass biological and non-biological family relationships. Genomic estimates of F, utilizing dense SNP data, are not subject to these limitations and are the most appropriate choice for the current study. Furthermore, Keller et al. [42] found F_{roh} to be more powerful than F_{ped} or marker-by-marker genomic estimates of F for detecting both recent and ancient parental relatedness, as well as being more highly correlated with the homozygous mutation load.

A secondary aim of this study was to investigate possible environmental cofactors that might contribute to the large excess of vulvar cancer cases found in the East Arnhem district. Smoking, herpes simplex

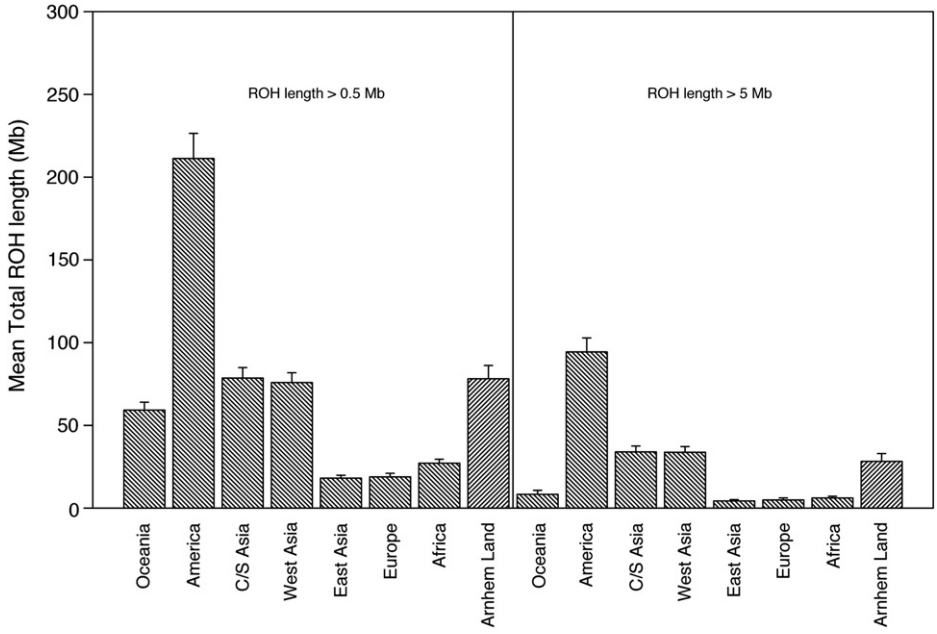

Fig. 2. Mean total length (and standard error) of the genome in ROH of >0.5 Mb and >5 Mb for the East Arnhem and continental HGDP population samples.

virus 2 (HSV2), gonorrhea and previous diagnosis of CIN have been found to be associated with increased risk of vulvar cancer [9–12], and trichomoniasis and HSV are associated with CIN [43]. The association with previous diagnosis of CIN is particularly well described, and is likely a consequence of the aetiological role of HPV in both diseases. This study found further evidence supporting an association between previous or concurrent diagnosis of CIN and diagnosis of vulvar cancer or VIN, but found no association with any other medical history variable. It is worth noting, however, that the extremely high rates of smoking and STIs in this population may obfuscate any potential association between these factors and vulvar cancer [13,44,45].

This aspect of the study was limited by several factors. First, the sample size was relatively small, which was a function of the limited number of cases available for recruitment. Secondly, history of STIs and CIN was collected from community health center records, which were largely paper based and may be incomplete. Further, cases were more likely to have undergone more frequent STI testing as a result of increased contact with health service providers, although controls were preferentially selected if there was evidence that they had participated in a recent Well Women's Screening health check, which includes STI testing among other health measures. Similarly, the smoking measure was relatively insensitive, as it was self-reported current practice for controls and practice at time of diagnosis for cases, although a systematic difference between reporting by cases and controls is unlikely. That the association with CIN remained statistically significant despite these limitations reinforces the importance of HPV in the etiology of vulvar cancer in this population.

Table 4
Top five consensus regions (by p-value) for association with case status.

Chromosome	Cases : controls sharing the region	Start position (Mb)	End position (Mb)	p-value	Genome- wide adjusted p-value
10	6 : 0	131.05	131.63	0.0009	1
17	5 : 0	44.81	45.33	0.0031	1
6	5 : 0	159.70	159.80	0.0031	1
6	5 : 0	128.53	129.82	0.0031	1
6	6 : 1	127.95	128.24	0.0047	1

This study found no evidence to suggest that autozygosity is a causal influence on the vulvar cancer cluster present in young Aboriginal women resident in East Arnhem, either through multiple recessive effects acting across the genome, or a single recessive effect of large effect size. This does not preclude the involvement of genetic risk factors in the etiology of vulvar cancer in this population; rather, analytical strategies designed to identify risk variants under alternative genetic models, such as a bottleneck effect causing a rare deleterious variant to become common in this population, may be more effective in this case. Although identifying the cause of this cluster is of immediate benefit to this small population, it is likely also to have wider ramifications for this rare and poorly understood disease, as well as potential relevance to other HPV-related anogenital neoplasia which appear to be increasing in incidence globally [46,47].

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.ygyno.2014.03.566.

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Conflict of interest statement

The authors declare that they have no conflict of interest.

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