Int. J. Cancer: **94,** 896–900 (2001)

Publication of the International Union Against Cancer

SHORT REPORT

HIGH FREQUENCY OF LOSS OF HETEROZYGOSITY IN VULVAL INTRAEPITHELIAL NEOPLASIA (VIN) IS ASSOCIATED WITH INVASIVE VULVAL SQUAMOUS CELL CARCINOMA (VSCC)

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Vulval intraepithelial neoplasia (VIN) is thought to be the premalignant phase of human papillomavirus (HPV)-associated vulval squamous cell carcinoma (VSCC). Various molecular events have been suggested as markers for progression from VIN to VSCC, but loss of heterozygosity (LOH) in vulval neoplasia has rarely been studied in this context. We performed LOH analysis by polymerase chain reaction (PCR) amplification of polymorphic microsatellite markers at 6 chromosomal loci (17p13-p53, 9p21-p16, 3p25, 4q21, 5p14 and 11p15). The presence of HPV was assessed using consensus PCR primers and DNA sequencing. To examine any association between LOH and the presence of invasive disease, we analyzed 43 cases of lone VIN III, 42 cases of lone VSCC and 21 cases of VIN with concurrent VSCC. HPV DNA was detected in 95% of lone VIN III samples and 71% of lone VSCC samples. Fractional regional allelic loss (FRL) in VIN associated with VSCC was higher than in lone VIN (mean FRL 0.43 *vs.* 0.21, $p < 0.005$). LOH at 3p25 occurred significantly more frequently in HPV-negative VSCC than in HPV-positive VSCC (58% *vs.* 22%, $p < 0.04$). These data suggest that genetic instability in VIN, reflected by LOH, may increase the risk of invasion. In addition, molecular events differ in HPV-positive and -negative VSCC and 3p25 may be the site of a tumor suppressor gene involved in HPV-independent vulval carcinogenesis.

Key words: *VIN; vulval; cancer; LOH; HPV*

Vulval intraepithelial neoplasia (VIN) is thought to be the premalignant phase of invasive vulval squamous cell carcinoma (VSCC). This hypothesis is based on the observation that VIN frequently occurs adjacent to VSCC,[1] that VIN and a subgroup of VSCC are associated with similar risk factors [smoking,[2] immunosuppression[3] and human papillomavirus (HPV) infection[2,4]] and that VIN is a monoclonal neoplastic condition.[5] The risk of progression of VIN to VSCC is unclear.[6,7]

Limited evidence is available about molecular events in vulval carcinogenesis. Loss of heterozygosity (LOH) is a common molecular event in malignancy, but has been studied only in relatively small numbers of VIN and VSCC.[8–11] We set out to document the LOH rates in VIN and VSCC in a larger series, to examine the relationship between the 2 conditions. Events common to both conditions could be early events in vulval carcinogenesis. Events occurring in VIN associated with VSCC but not in lone VIN could be markers for risk of progression to VSCC.

Because human papillomavirus (HPV) is thought to be involved in the development of VIN-associated VSCC, but is not found so often in VSCC occurring in the absence of VIN,[2] we also performed HPV analysis, to compare LOH in HPV-positive and HPV-negative VSCC.

MATERIAL AND METHODS

Samples

Patients with VIN and VSCC diagnosed between 1989 and 1997 were identified using the computerized database of the pathology departments of St. Bartholomew's and the Royal London Hospitals. Samples containing both normal and neoplastic tissue were as follows: 43 cases of VIN III alone, 42 cases of VSCC alone and 21 cases of VIN associated with concurrent VSCC (18 of which had the concurrent VSCC still remaining on the specimen blocks after serial sectioning). Of the 60 VSCC cases, 24 were stage I, 11 were stage II, 9 were stage III, 3 were stage IV and in 13 information for accurate staging was not available. Of the 21 cases of VIN associated with VSCC, 18 were VIN III, 2 were VIN II and 1 was VIN I. The following other epithelial abnormalities were observed: squamous cell hyperplasia (9 cases), lichen sclerosus (7 cases), lichen planus (1 case) and Paget's disease (1 case). The relevant paraffin-embedded tissue samples underwent serial sectioning as follows: one 4-μm section was mounted, stained with hematoxylin and eosin (H&E), covered and used as a reference slide. One 10-μm section was also stained with H&E, but left uncovered for microdissection. For clarity, the VIN and VSCC samples from the 21 patients with a concurrent diagnosis of both conditions will henceforth be termed "VIN(+)" and "VSCC(+)," respectively. VIN samples from patients without associated VSCC will therefore be termed "VIN(−)" and VSCC samples from patients without associated VIN will be termed "VSCC(−)".

Microdissection of tissue samples and extraction of DNA

The uncovered H&E-stained 10-μm section was mounted on a dissecting microscope and compared with the reference slide. Areas of tissue containing >70% VSCC, >70% VIN or 100% normal cells were identified and microdissected. The tissue was placed in 100 μl of 10% Chelex chelating resin (Sigma, St. Louis, MO) in distilled water; then 1 μl of 20 mg/ml proteinase K was added to the tube, which was vortexed, placed in a shaking water bath at 56°C for 30 min and boiled for 8 min. The sample was

Grant sponsor: Gynaecology Cancer Research Fund; Grant sponsor: Stroyberg Vagn Jensen Foundation.

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Received 10 January 2001; Revised 11 May 2001; Accepted 25 May 2001

TABLE I – LOCI STUDIED, PRIMER PAIRS, ANNEALING TEMPERATURES AND MAGNESIUM CONCENTRATIONS

Chromosomal location	Primer	Primer pair sequences	Annealing temperature (°C)	Mg^{2+} (mM)
17p13.1 (p53)	TP53	AGGGATACTATTCAGCCCCGAGGTG ACTGCCACTCCTTGCCCCATTC	58	3.5
9p21 (p16)	D9S171	AGCTAAGTGAACCTCATCTCTGTC ACCCTAGCACTGATGGTATAGTCT	55	5.0
3p25	D3S1360	GACCCCGGGCTCCACAGCAAA CCTGGCCTCTTCGGGGTGAC	60	3.5
4q21-23	D4S2458	GGCACATGAAAATGGCGAATG TTCCTTCCCCTGTAGCTGCCC	55	3.5
5p14-13	D5S661	AGAATGTTTCCTGTGTATGTGC CAAAATCCCAATGTCTCTGC	55	5.0
11p15.5	D11S922	GGGGCATCTTTGGCTA TCCGGTTTGGTTCAGG	55	4.5

centrifuged at 10,000*g* for 10 min to pellet any remaining debris, then 1–5 μl of the supernatant was used directly in the polymerase chain reaction (PCR).

Amplification of polymorphic microsatellite markers

PCR was performed in a volume of 20 μl, containing approximately 20–100 ng DNA, 0.2 mM deoxyribonucleotide triphosphates, 0.25 U of Taq supreme DNA polymerase (Hellena Biosciences, Sunderland, UK), 1× buffer (supplied with enzyme), 1.5 pmol of forward primer, 2 pmol of reverse primer and 0.5 pmol of ^{32}P-labeled forward primer. PCR was performed in a Touchdown Thermal Cycler (Hybaid, Ashford, UK). Table I shows the chromosomal loci, primer pairs used to study amplify polymorphic repeats, magnesium concentrations and annealing temperatures. All PCR reactions were prepared according to the following protocol: DNA denaturation for 5 min at 95°C, hot start at 85°C, 35 cycles of denaturation at 95°C for 40 sec (ramped at 1°C/sec, obviating the requirement for a separate extension step) and 30 sec at the annealing temperature. This procedure was followed by a final extension step at 72°C for 5 min.

Separation, visualization and interpretation of LOH analysis PCR products

The PCR products were electrophoresed on a 5% denaturing polyacrylamide gel. Autoradiography was performed. LOH was scored as complete loss of 1 allele or >50% reduction in intensity of 1 of the alleles from the neoplastic tissue relative to the normal tissue of the same patient.[9] All autoradiographs were read by 2 individuals (A.N.R., A.R.) who were blinded as to the histologic type of the samples. Examples are shown in Figure 1.

Detection and sequencing of HPV DNA

PCR volume and concentrations were as above, but we used 20 pmol each of consensus genital type HPV L1 gene primers GP5+ and GP6+ (according to Kohlberger *et al.*[4]) and 3.5 mM Mg^{2+}. PCR was performed using a 3-min denaturation step at 94°C followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 2 min and extension at 72°C for 1.5 min. A final extension step at 72°C for 7 min was performed. HeLa cell DNA was used as the positive control and cross-contamination was checked for using water controls. PCR products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide staining and ultraviolet illumination. The amplification product from the above reaction was cut out of the agarose gel using a sterile scalpel blade. DNA was extracted from the gel using a commercial kit (QIAquick Gel Extraction Kit, Qiagen, Crawley, UK), following manufacturer's instructions. DNA sequencing was performed using a commercial kit (ABI Prism dRhodamine Terminator Cycle Sequencing Ready Reaction Kit, PE Biosystems, Warrington, UK), following manufacturer's instructions. Samples were analyzed on an ABI Prism 377 automatic sequencer (PE Biosystems). Data was analyzed using the manufacturer's software and the output was entered onto the National Center for Biotechnology Information BLAST search facility (http://www.ncbi.nlm.nih.gov/BLAST/) for sequence comparison with known HPV types. Sequences with a greater than 90% match to a known type were classified as that type. Sequences with less than 90% match were classified as unknown type.

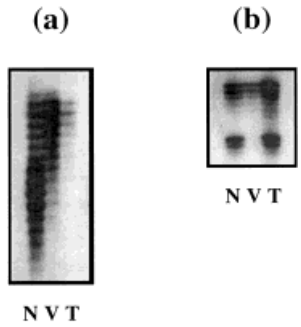

FIGURE 1 – Autoradiographs showing examples of loss of heterozygosity in vulval intraepithelial neoplasia (VIN) and/or concurrent vulval squamous cell carcinoma (VSCC). N, normal tissue; V, VIN; T, VSCC. (*a*) Loss of upper allele at p53 locus in VIN and VSCC from same patient. (*b*) Loss of lower allele at p16 locus in VIN, but retention of lower allele in VSCC from same patient.

Age-matching of comparison groups

Where appropriate, samples from one group were matched with samples from a comparison group, such that the patients' ages fell within ±3 years of the age of the sample with which it was being compared. Unmatched samples were excluded from the analysis.

Statistical analysis

Proportions of informative samples showing LOH at individual loci were compared using Fisher's exact test or χ^2 test, where appropriate. To take into account the differing proportions of noninformative cases in the different sample groups, we calculated the fractional regional allelic loss (FRL) for each sample.[12] FRL for each sample was the total number of loci undergoing LOH/total number of informative loci. FRL scores for sample groups were compared using the nonparametric Wilcoxon test. Ages of different sample groups were compared overall using 1-way analysis of variance and the Bonferroni post hoc test for comparisons between individual groups. Stage distribution between groups was compared using the Kolmogorov-Smirnov test. Significance was taken at the 5% level.

RESULTS

DNA was successfully extracted and amplified with all primers from all samples. Proportions of informative samples showing LOH at individual loci, according to sample group, are shown in

Figure 2. The number of samples in each group, median age and age ranges are shown in Table II. FRL in VIN(+) was significantly greater than in VIN(−) (mean FRL 0.43 *vs.* 0.21, $p < 0.005$). Seventeen of 21 VIN(+) samples were successfully age-matched with a VIN(−) sample. FRL in age-matched VIN(+) was significantly greater than in age-matched VIN(−) (mean 0.35 *vs.* 0.16, $p < 0.03$). FRL in VIN(+) was significantly greater than in VSCC(+) (mean FRL 0.43 *vs.* 0.21, $p < 0.02$). This finding was surprising as VSCC(+) would be expected to have arisen from adjacent VIN(+) and therefore losses occurring in the VIN should also occur in the VSCC. A possible explanation for this discrepancy was contamination of VSCC(+) samples by nonneoplastic stromal cells or infiltrating lymphocytes.

We scored the samples for lymphocytic infiltration and the presence of stromal cells within VSCC(+) using a 6-point scale: up to 3 points for lymphocytic infiltration and up to 3 points for stromal contamination (data not shown). Scoring was performed by 1 individual (D.H.) who was blinded as to whether samples were VSCC(+) or VSCC(−). The maximum contamination exhibited by any sample was 4 out of a possible 6. Five of 9 VSCC(+) samples showing LOH at 1 or more locus scored 4 on this scale.

The p16 locus was lost significantly more in VIN(+) than in VIN(−) ($p < 0.05$). The frequency of LOH at the p53 locus was higher in VSCC(+) (33%) than in VIN(+) (23%) and VIN(−) (17%), but these differences were not statistically significant.

Overall, there were significant differences between the ages of the sample groups (Table II) ($p < 0.001$); patients in the VIN(+), VSCC(+) and VSCC(−) groups were all significantly older than those in the VIN(−) group. No other age comparisons were significant.

Forty-one of 43 (95%) VIN(−) samples, 30 of 42 (71%) VSCC(−) samples and 17 of 18 (94%) VSCC(+) samples were HPV positive using consensus primers. Sequencing confirmed the presence of HPV DNA in 73 of 74 samples. The following HPV types were identified: 16 (92%), 33 (3%), 45 (1%), 11 (1%) and unknown type (3%). The unknown types displayed a 85–89% match to a known type. There was no significant difference in the stage distribution between HPV-positive and HPV-negative VSCC. Four of 13 HPV-negative VSCC were associated with lichen sclerosus compared with 3 of 47 HPV-positive VSCC ($p < 0.04$).

The frequency of LOH at the 6 loci studied in HPV-positive VIN and HPV-positive and -negative VSCC are shown in Figure 3. FRL in HPV-negative VSCC compared with HPV-positive VSCC did not differ significantly (mean FRL 0.33 vs. 0.28, $p < 0.56$). LOH at 3p25 in HPV-negative VSCC occurred at a significantly higher frequency compared with HPV-positive VSCC (58% vs. 22%, $p < 0.04$). The frequencies of LOH at the p53, p16 and 11p15.5 loci were higher in HPV-positive VSCC than in HPV-positive VIN; however, these differences were not significant.

DISCUSSION

We wanted to document and compare LOH rates in lone VIN and VIN and VSCC occurring concurrently to assess possible markers for progression from VIN to VSCC. Comparison of LOH in HPV-positive and -negative VSCC might provide insight into the pathways of vulval carcinogenesis. There have been 4 previous reports of LOH in vulval neoplasia. The first[8] studied 4 cases of VSCC, 2 of which had adjacent VIN. This analysis was aimed at investigating the clonal evolution of these conditions, rather than documenting overall rates of LOH. The other studies[9–11] examined HPV status and LOH at multiple chromosomal loci in 16 cases of

TABLE II – SAMPLE TYPE AND NUMBERS, ABBREVIATIONS, MEDIAN AGE RANGES AND AGE OF DIFFERENT SAMPLE GROUPS

Sample type (*n*)	Abbreviation	Median age (range), years
VIN III not associated with VSCC (43)	VIN(−)	46 (27–83)
VSCC not associated with VIN (42)	VSCC(−)	75 (21–94)
VSCC associated with VIN[1] (18)	VSCC(+)	63 (35–88)
VIN associated with VSCC[1] (21)	VIN(+)	63 (35–88)

VIN, vulval intraepithelial neoplasia; VSCC, vulval squamous cell carcinoma.–[1]These samples are from the same 21 patients, but the VSCC could not be analyzed in 3 cases in which serial sectioning had removed the VSCC from the specimen block.

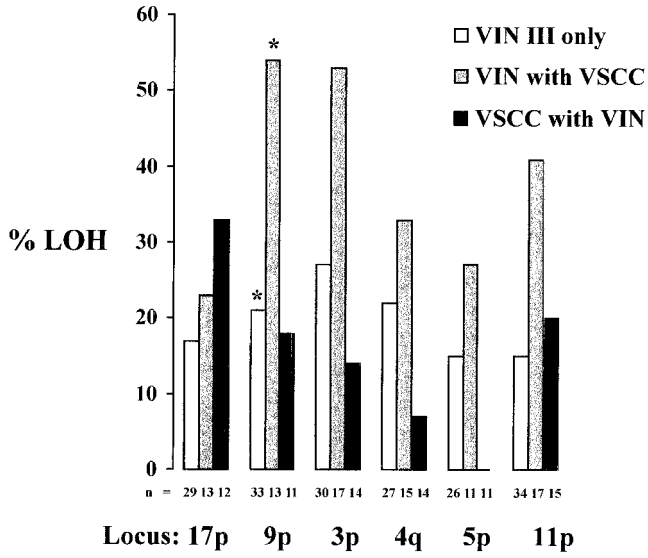

FIGURE 2 – Proportion of informative samples undergoing loss of heterozygosity (LOH) at 6 loci in VIN(−), VIN(+) and VSCC(+). *Significant differences between groups ($p < 0.02$). n, number of informative results in each column; VIN, vulval intraepithelial neoplasia; VSCC, vulval squamous cell carcinoma.

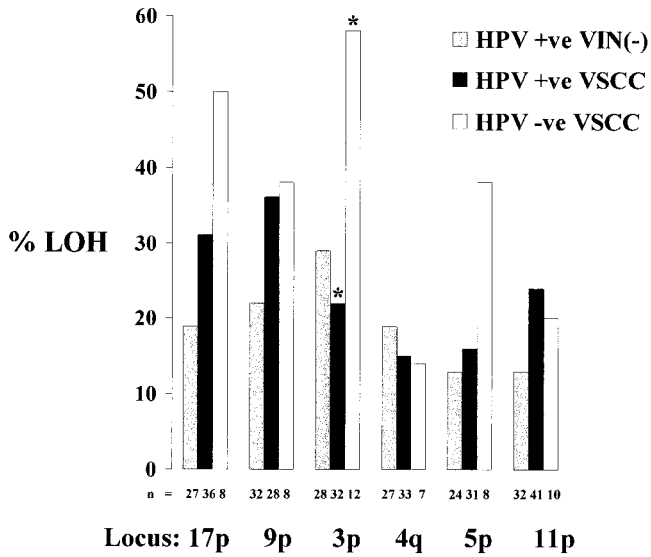

FIGURE 3 – Proportion of informative samples undergoing loss of heterozygosity (LOH) at 6 loci in HPV-positive VIN(−), HPV-positive VSCC and HPV-negative VSCC. *Significant differences between groups ($p < 0.04$). n, number of informative results in each column; HPV, human papillomavirus; VIN, vulval intraepithelial neoplasia; VSCC, vulval squamous cell carcinoma; +ve, positive; -ve, negative.

VSCC, 30 cases of VSCC (and associated VIN) and 30 cases of VIN, respectively. We undertook a larger study, which analyzed VIN and VSCC samples in an attempt to establish whether HPV-positive and -negative VSCC undergo different chromosomal losses and whether the VIN associated with invasive disease differs from lone VIN.

The loci studied were chosen for specific reasons: 17p13 because it harbors p53, which accumulates in 53–68% of VSCC[13,14] and 9p21 (p16 gene) and 3p25 because they frequently exhibit LOH in squamous head and neck cancer.[15–17] p16 is also disrupted in VIN and VSCC.[18] The other loci studied (4q21, 5p14, 11p15) may be involved in cervical cancer.[19]

Clear losses were visible in VIN, suggesting that VIN is a clonal neoplasm. LOH was frequent in both lone VIN and VSCC (53% and 81% at 1 or more locus, respectively), suggesting that VIN, like cervical intraepithelial neoplasia (CIN),[20] frequently undergoes LOH which may alter tumor suppressor gene function.

HPV is implicated in CIN and cervical squamous cell carcinoma (CxSCC) as well as VIN and some VSCC.[2,4,21] LOH was observed in HPV-positive VIN and VSCC at loci frequently lost in CxSCC (4q21, 5p14, 11p15), but at lower rates.[19] Comparison with the other studies in vulval neoplasia is difficult because most of the microsatellite markers used differed from the present study. However, the largest of the previous studies of VSCC[10] found a significantly higher overall frequency of LOH at 7 3p loci in HPV-negative compared with HPV-positive VSCC, although the individual difference at 3p25 did not reach significance. Both of the previous VSCC studies[9,10] reported (nonsignificantly) higher LOH rates at 17p in HPV-negative VSCC compared with HPV-positive VSCC. With regards to VIN, comparison with 1 of the 2 previous studies[11] is impossible, as the frequencies of LOH at individual loci were amalgamated with results from nonneoplastic epithelial disorders. The other study[10] found a similarly low frequency of LOH at the p53 locus in VIN(+); however, that study reported 0% LOH at 3p25 in VIN(+) compared with 53% in our study. This difference may relate to sample size, as only 7 VIN II/III samples in the previous study were informative.

FRL in VIN(+) was significantly higher than in VIN(−), suggesting that genetic instability in VIN may predispose to invasion. In cervical neoplasia, microsatellite instability (another measure of genetic instability) was found significantly more in intraepithelial lesions associated with invasive disease than in lone intraepithelial lesions.[22] It could be argued that this difference in FRL in our study resulted from the median age of patients in the VIN(+) group being older than those in the VIN(−) group, implying that greater LOH in VIN(+) reflects the age of the VIN and the longer it persists, the higher the chance of developing cancer. However, the difference in FRL remained even when we corrected for patient age. Although this finding cannot entirely exclude the possibility that the VIN(+) cases have existed for longer than the VIN(−) cases, it supports the argument that the differences in FRL reflect inherent genetic instability, rather than instability that has accumulated with time.

The finding of significantly higher FRL in VIN(+) than in VSCC(+) from the same patients is perplexing. This phenomenon was observed in 1 of the 2 cases of concurrent VIN and VSCC in another study[8] and is exemplified in Figure 1*b*, in which the VIN(+) has lost an allele at a locus not lost in the adjacent VSCC(+). One possible explanation is that VSCC is inherently more likely than VIN to be contaminated with nonneoplastic stromal cells or lymphocytes, which could swamp out the neoplastic DNA in the assay, thus masking LOH. We therefore scored samples for contamination. Five of 9 VSCC(+) samples exhibiting LOH had maximal contamination.

This finding suggested that we had successfully isolated tumor DNA from less contaminated areas on the slides or that amplification of tumor DNA was sufficient to swamp out any normal DNA. Furthermore, if VSCC were more contaminated than VIN, then VSCC would be expected to demonstrate allelic imbalance (classified as 50–99% reduction in intensity of 1 allele), rather than pure LOH (100% loss of 1 allele). The proportion of VSCC samples demonstrating allelic imbalance, rather than pure LOH, was lower than in VIN cases (38% vs. 51%, data not shown). This finding suggests that microdissection of VSCC samples produced cell populations at least as pure as those from VIN samples. We therefore conclude that the finding of higher FRL in VIN(+) than VSCC(+) is real. This finding suggests that some of the losses observed in VIN(+) occurred after the point at which a subclone of that VIN had acquired the malignant phenotype. We therefore speculate that inherently unstable VIN gives rise to the invasive phenotype, which then selects for LOH at loci different to those which the unstable VIN continues to lose, either as a random consequence of genetic instability, or as it evolves down a different path from the VSCC. This theory fits the observations at the p53 locus, which, unlike the other loci studied, was lost more in VSCC(+) than VIN(+) (Fig. 2). In a similar study of cervical cancer[22] Chu *et al*. observed microsatellite instability in some cases of intraepithelial neoplasia, but not in the associated invasive disease. This evidence supports the hypothesis of clonal evolution in intraepithelial neoplasia and possibly the selection of a more stable clone in invasive disease.

Disruption of p16 or RB has been found more in VSCC (72%, $n = 32$) than in VIN (60%, $n = 10$).[18] In our series, the p16 locus (9p21) was lost significantly more in VIN(+) than in VIN(−) (Fig. 2). However, the lowest rate of p16 loss was observed in VSCC(+), therefore it seems unlikely that loss of p16 is a significant event in progression. Similar nonsignificant trends were observed with LOH at 3p25 and 11p15.5, so it also seems unlikely that these loci are involved in progression. This conclusion is reinforced by the relatively low frequency of LOH at these loci in HPV-positive VSCC (Fig. 3). More probably, these losses reflect increased genetic instability in the VIN(+) samples.

p53 may be involved in progression from VIN to VSCC as p53 product accumulated in 53% of VSCCs ($n = 66$)[13] and 44% ($n = 34$)[14] of VIN associated with VSCC, but 0% of VIN not associated with VSCC ($n = 28$).[4] In our series, the p53 locus was lost more in VSCC(+) than in VIN(+), which in turn had more loss of p53 than VIN(−) (Fig. 2), but these differences were nonsignificant. HPV-positive VSCC lost p53 more often than HPV-positive VIN (Fig. 3), but the difference was not significant. Given these results and the fact that LOH at p53 occurred in only 31% of HPV-positive VSCC, it seems unlikely that LOH at p53 is involved in the progression of VIN to VSCC. However, there does appear to be a possible role for LOH at the p53 locus in HPV-negative VSCC, as 50% of informative samples lost this locus (Fig. 3). This hypothesis concurs with another study that found accumulation of p53 product in 53% of HPV-negative VSCC ($n = 54$).[13] The observation that 58% of HPV-negative VSCC underwent LOH at 3p25 suggests that this locus may be the site of a tumor suppressor involved in an HPV-independent pathway of vulval carcinogenesis. The fact that this locus was lost significantly more in HPV-negative VSCC than in HPV-positive VSCC (Fig. 3) supports dual etiologies in VSCC.

In conclusion, we found that genetic instability in VIN, reflected by LOH, is associated with invasive disease. The higher rate of LOH at 3p25 in HPV-negative compared with HPV-positive VSCC supports the hypothesis that there are 2 different etiologies in vulval carcinogenesis and implicates this locus as the site of a possible tumor suppressor in an HPV-independent pathway.

ACKNOWLEDGEMENTS

We thank Alex Brown and his staff for cutting and mounting the tissue slides; Steve Jones for providing the list of patients with VIN and VSCC; Janice Thomas, Melvyn Carroll and Philippe Van Trappen for assisting with the statistical analysis and Judy Breuer for providing the sequencing facilities. A.N.R. was supported by the Gynaecology Cancer Research Fund; A.R. was supported by the Stroyberg Vagn Jensen foundation.

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