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Monoclonal Origin of Vulvar Intraepithelial Neoplasia and Some Vulvar Hyperplasias

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Squamous neoplasms of the female genital tract, including vulvar intraepithelial neoplasia, presumably are derived from a single cell. This study addressed this hypothesis and determined the clonal status of other squamous epithelial alterations associated with vulvar carcinoma, including hyperplasia and lichen sclerosis. X chromosome inactivation patterns of 22 epithelial lesions and matched normal epithelium were determined using a polymerase chain reaction (PCR)-based assay targeting the X-linked human androgen receptor gene (HUMARA). Clonality was inferred by comparing matched lesional and control tissues as follows: 1) monoclonal, if intensity of either PCR product was skewed relative to normal reference epithelium (control), 2) polyclonal, if both lesional and control were unskewed, and 3) unknown, if both lesion and control tissues were skewed toward the same allele. Two cases were excluded because of noninformative homozygous HUMARA alleles. Of 8 vulvar intraepithelial neoplasias analyzed, 7 were scored monoclonal and 1 polyclonal. Of 12 hyperplasias, 6 were monoclonal, including one with lichen sclerosis, 2 were polyclonal, and in 4, the clonal status could not be determined. The PCR-based clonal assay supports a monoclonal derivation for vulvar intraepithelial neoplasia and, in some cases, vulvar hyperplasia, and lichen sclerosis. The finding of monoclonal hyperplasia and lichen sclerosis suggests that clonal expansion may evolve before the development of morphological atypia in these epithelia. (Am J Pathol 1997, 150:315–322)

Vulvar cancer is a relatively uncommon disease with an incidence of approximately 1.8 per 100,000,[1–3] or approximately one-eighth that of cervical carcinoma.[4–6] Epidemiological and molecular studies indicate a diverse pathogenesis, with risk factors for vulvar cancer including vulvar inflammatory disorders (lichen sclerosis and hyperplasia) and human papillomaviruses (HPVs).[7–12] Approximately one-third of cases are associated with a classic vulvar intraepithelial neoplasia (VIN; carcinoma *in situ* or Bowen's disease), another one-third are associated with differentiated variants of VIN (differentiated carcinoma *in situ*), and the remainder develop in the setting of benign-appearing squamous epithelium, including hyperplasia and lichen sclerosis.[13,14] The pathogenesis of classic forms of VIN is closely related to HPV infections, predominatly HPV type 16.[14] The mechanisms by which differentiated VIN and benign squamous changes evolve into carcinoma are unclear but probably involve sequential changes in the epithelium produced by endogenous (host genetic) cellular events, with HPV infection being rarely found.[13–15]

It is presumed generally that squamous cancer precursor lesions of the female genital tract are monoclonal in origin. Determining a single cell of origin in genital tract squamous neoplasms has usually entailed identifying common molecular markers, such as common chromosomal integration sites in papillomaviruses[16] or X-linked enzyme markers (glucose-6-phosphate-dehydrogenase) that permitted assessment of clonality in tissues from women who were heterozygous for the allele.[17–19] More recently, molecular-based clonal assays targeting inactivation of the androgen receptor gene on the X chromosome have offered greater flexibility, with applications to both fresh and archival specimens. The determination of a clonal origin for tissues is based on three established phenomena[20]: 1) only one X chromo-

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some is active in each female cell, as determined early in embryogenesis, and is stable for all subsequent cell divisions, 2) activation is associated with consistent changes in methylation of cytosine residues at some sites, and 3) the alleles of a locus can be distinguished at the DNA level. Allen et al[21] identified a region of the androgen receptor gene on the X chromosome that is highly polymorphic (polymorphism information content = 0.9, with 20 known alleles) for the number of CAG repeats. These repeats can vary from 11 to 31 copies and can be distinguished by sizing of polymerase chain reaction (PCR) amplimers from primers flanking the length polymorphism. Furthermore, inactivation-linked methylation can be recognized at *Hha*I restriction sites, which are methyl sensitive in their cleavage specificity.

The purpose of this study was to determine 1) whether VINs scored as monoclonal proliferations by the PCR-based assay for X inactivation and 2) whether similar alterations in clonal status occurred with other cellular alterations associated with cases of invasive carcinoma.

Materials and Methods

Case Selection

Cases of archival tissues were selected after histological review. Briefly, frozen or formalin-fixed, paraffin-embedded tissue were serially sectioned at 20 μm and placed on glass slides. For each specimen, one slide was stained to identify the region of epithelial lesion, and four to eight sections were carefully trimmed to remove only affected tissue to a sterile Eppendorf tube. The method used for clonal assessment in this study was essentially that of Allen et al[21] with some modifications. DNA was extracted by overnight digestion at 62°C with proteinase K and carrier glycogen. Digested DNA was extracted once each with phenol, phenol/chloroform, and chloroform and purified with Gene Clean (Bio 101, La Jolla, CA). After resuspending samples in 1× *Hha*I digestion buffer (New England Biolabs, Beverly, MA), one-half of each sample was transferred to a fresh tube and 10 U of *Hha*I enzyme was added. Both sets of samples (with or without *Hha*I) were incubated for 2 hours at 37°C.

After restriction digestion, both sets of samples were boiled for 10 minutes to denature the enzyme and 2 to 5 μl of each sample was amplified in a PCR. Primers (0.5 μmol/L each) ANR1 (5′-CCTACCGAGGAGCTTTCCAGAATCT) and ANR2 (5′-GCTG TGAAGGTTGCTGTTCCTCAT) were used under standard conditions (Perkin-Elmer, Norwalk, CT), except dGTP was replaced with 7-deaza-2′-dGTP[22] to improve polymerase reading through the GC-rich repeat region and 2 μCi of [α^{32}P]dCTP (3000 Ci/mmole; New England Nuclear, Boston MA) was added per 25 μl reaction.[23] PCR parameters were as follows: initial 5-minute denaturation followed by 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute, followed by a 5-minute extension at 72°C. PCR products were loaded on nondenaturing 5.5% polyacrylamide gels and electrophoresed overnight at 4 V/cm. Gels were dried and exposed to autoradiography film.

X chromosome inactivation was assessed by comparing PCR products from the hypervariable region with and without predigestion with *Hha*I. A case was scored as monoclonal if a conspicuous shift in band intensity of the higher or lower molecular weight product (skewing) occurred in the digested/amplified product of the lesional *versus* adjacent normal control epithelium. Cases in which lesional and normal epithelium both had balanced X inactivation were scored as polyclonal. Cases in which similarly skewed X inactivation was seen in lesional and normal tissues were interpreted as noninformative.

Results

A total of 26 cases were evaluated, including 4 individuals with no disease, 9 with VIN, and 13 with hyperplasia/lichen sclerosis. Of 6 reference epithelia from the 4 normals, 5 exhibited no skewing of product intensities (Table 1), and 1 exhibited skewing toward the lower molecular weight allele. In 2 of the 22 cases with an epithelial abnormality, amplification of the hypervariable region produced a single product, indicating an identical number of trinucleotide repeats on the two alleles (homozygosity). Table 1 summarizes the analysis of the remaining 20 cases. Of 8 VINs analyzed, 7 exhibited a single or predominantly single (skewed) product after enzymatic digestion and PCR amplification, which was interpreted as monoclonality when compared with reference tissue (Figure 1). In 1 case, skewing was not identified and the clonality was scored as unknown (Table 1, case 1). Potential explanations for this unexpected finding included a change in methylation status with microsatellite instability, a numerical chromosome anomaly, technical reasons (ie, contamination with polyclonal tissue), and a polyclonal VIN.

Of 12 cases of hyperplasia (including lichen sclerosis), 6 were scored as monoclonal, including 1

Table 1. *Histological Diagnosis and Clonal Status of Intraepithelial Lesions of the Vulva*

Case	Diagnosis	Number of assays	Skewing (observed)	Clonality (inferred)
Normal tissue controls				
1	Normal	2	Unskewed	Reference*
2a	Normal	1	Unskewed	Reference
2b	Normal	1	Unskewed	Reference
3	Normal	1	Lower allele	Reference
4a	Normal	1	Unskewed	Reference
4b	Normal	1	Unskewed	Reference
Vulvar intraepithelial neoplasia				
1	Normal	2	Unskewed	Reference*
	VIN	3	Unskewed	Polyclonal†
2	Normal	1	Lower allele	Reference
	VIN	1	Upper allele	Monoclonal
3	Normal	1	None	Reference
	VIN	1	Upper allele	Monoclonal
4	Normal	1	Lower allele	Reference
	VIN	1	Upper allele	Monoclonal
5	Normal	1	Unskewed	Reference
	VIN	1	Lower allele	Monoclonal
6	Normal	2	Unskewed	Reference
	VIN	2	Lower allele	Monoclonal
7	Normal	1	Lower allele	Reference
	VIN	1	Upper allele	Monoclonal
8	Normal	2	None	Reference
	VIN	2	Upper allele	Monoclonal
Hyperplasia/lichen sclerosis				
1	Normal	1	Lower allele	Reference*
	Hyperplasia	2	Lower allele	Unknown‡
2	Normal	4	Unskewed	Reference
	LSA	3	Upper allele	Monoclonal
	LSA/hyperplasia	7	Upper allele	Monoclonal
3	Normal	2	Unskewed	Reference
	Hyperplasia	1	Unskewed	Polyclonal
4	Normal	1	Unskewed	Reference
	Hyperplasia	2	Unskewed	Polyclonal
5	Normal	1	Lower allele	Reference
	Hyperplasia	1	Lower allele	Unknown‡
6	Normal	1	Upper allele	Reference
	Hyperplasia	1	Upper allele	Unknown‡
7	Normal	1	Unskewed	Reference
	Hyperplasia	1	Lower allele	Monoclonal
8	Normal	1	Unskewed	Reference
	Hyperplasia	1	Lower allele	Monoclonal
9	Normal	1	Lower allele	Reference
	Hyperplasia	1	Unskewed	Monoclonal
10	Normal	2	Unskewed	Reference
	Hyperplasia	2	New allele§	Monoclonal§
	Hyperplasia	2	New allele§	Monoclonal§
11	Normal	2	Unskewed	Reference
	Hyperplasia	2	Lower allele	Monoclonal
12	Normal	2	Lower allele	Reference
	Hyperplasia	2	Lower allele	Unknown‡

LSA, lichen sclerosis.
*Serves as reference tissue and is not scored for clonality.
†Scored as polyclonal; other potential explanations are discussed in the text.
‡Clonality cannot be assessed; skewing in *Hha*I predigested samples in both normal and lesion-derived samples.
§A third band was identified in the hyperplasia samples and interpreted as evidence of monoclonality (see text).

case (case 2) in which skewing was identified in epithelium containing lichen sclerosis and hyperplasia (Figure 2). Included also was a case in which a band not present in normal tissue was identified in the hyperplasia, consistent with *de novo* appearance of a novel allele such as those seen in microsatellite instability. The band intensity of novel and constitutive alleles was similar, suggesting true emergence of an altered HUMARA gene rather than PCR artifact. Just 2 cases exhibited no skewing and were scored as polyclonal, and 4 exhibited skewing of both lesional and normal tissue.

We do not believe that observed allelic skewing was caused by technical artifacts. Conditions of bi-

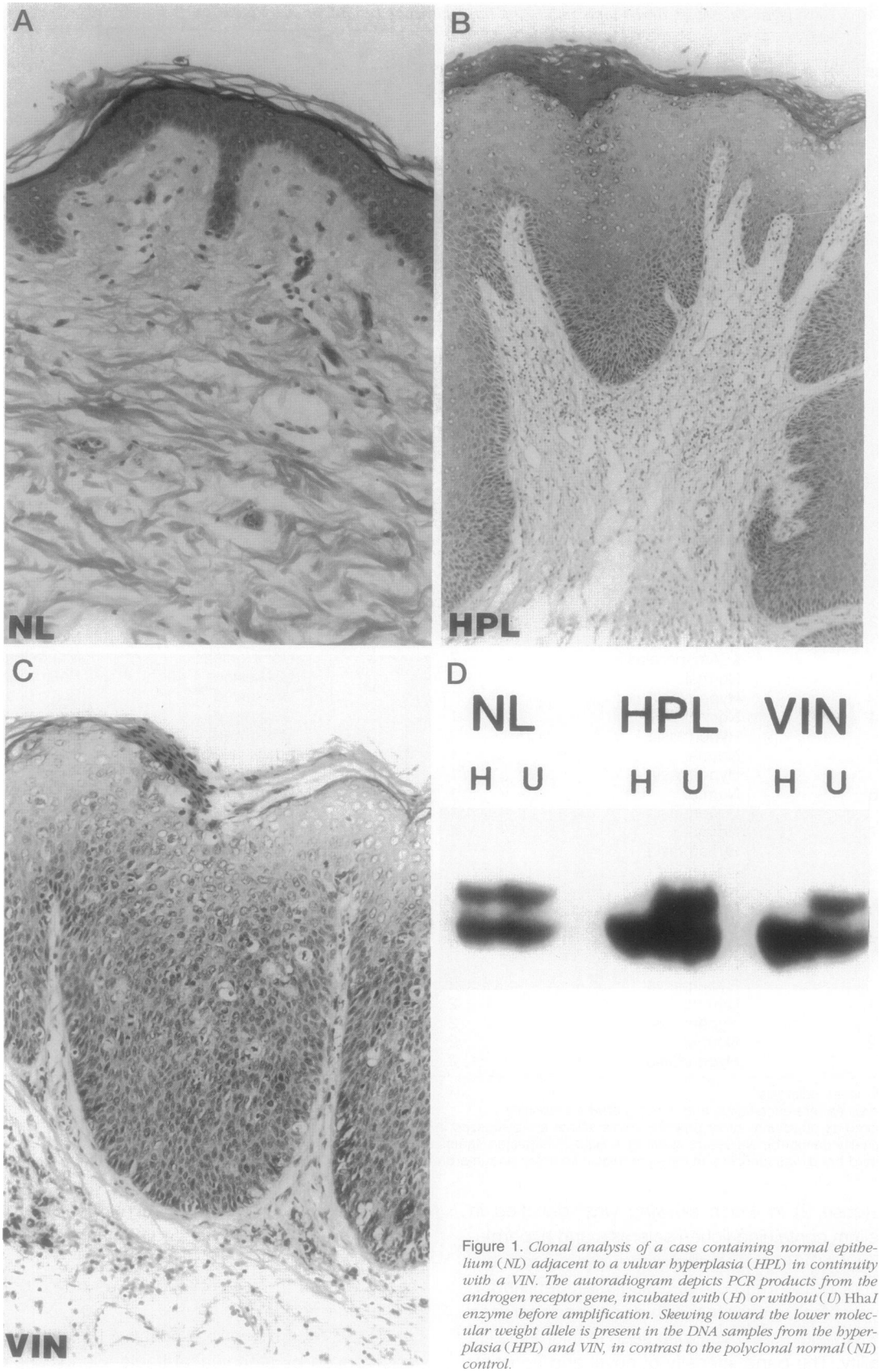

Figure 1. *Clonal analysis of a case containing normal epithelium (NL) adjacent to a vulvar hyperplasia (HPL) in continuity with a VIN. The autoradiogram depicts PCR products from the androgen receptor gene, incubated with (H) or without (U)* Hha*I enzyme before amplification. Skewing toward the lower molecular weight allele is present in the DNA samples from the hyperplasia (HPL) and VIN, in contrast to the polyclonal normal (NL) control.*

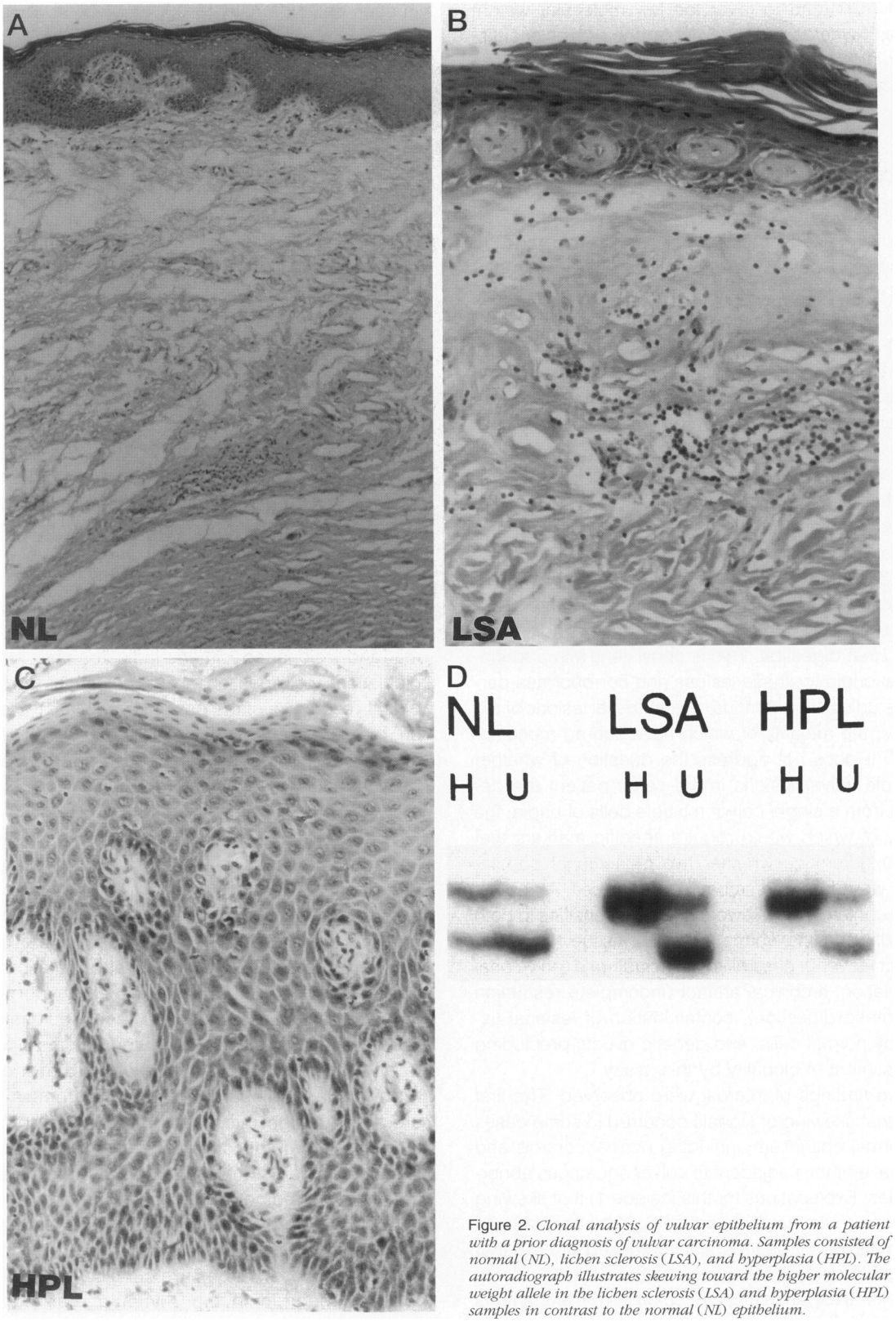

Figure 2. *Clonal analysis of vulvar epithelium from a patient with a prior diagnosis of vulvar carcinoma. Samples consisted of normal (NL), lichen sclerosis (LSA), and hyperplasia (HPL). The autoradiograph illustrates skewing toward the higher molecular weight allele in the lichen sclerosis (LSA) and hyperplasia (HPL) samples in contrast to the normal (NL) epithelium.*

ased PCR tend to favor the low molecular weight allele,[22] whereas the skewing in the monoclonal tissues in this study was equally distributed between the low and high molecular weight alleles.

Discussion

The emergence of a clonality assay with application to both unfixed and archival tissues has made it possible to determine whether clonal cell populations emerge before the acquisition of morphological alterations characteristic of neoplasia. This issue has been addressed in the endometrium, in which cytogenetic and clonal assays have determined that endometrial polyps and endometrial hyperplasias are clonal in origin.[23–25] In the case of the vulvar epithelium, the question we addressed concerned not only whether VIN lesions were monoclonal but also whether other abnormalities associated with carcinoma, including lichen sclerosis and hyperplasia, were monoclonal.

In this study, the majority (seven of eight) of informative cases of VIN heterozygous in the hypervariable region contained a predominant or single allele after *Hha*I digestion. This is consistent with a monoclonal origin for these lesions and corroborates parallel studies of squamous intraepithelial lesions of the cervix, the majority of which have scored monoclonal.[26] It does not address the question of whether multiple vulvar lesions in the same patient are derived from a single cell or multiple cells of origin, the study of which would require specific markers that would determine whether two monoclonal populations were from the same progenitor cell. An eighth case contained unskewed product intensities in both the VIN and the normal adjacent tissue. This may reflect several possibilities, including a polyclonal population, technical artifact (incomplete restriction enzymatic digestion), contamination of lesional tissue by normal cells, and genetic events precluding assessment of clonality by this assay.

Two findings of interest were observed. The first was that skewing of signals occurred in some cases of normal epithelium, including normal controls and normal epithelia adjacent to vulvar squamous abnormalities. Explanations for this include 1) that skewing may occur via technical artifacts, 2) that some polyclonal tissue by chance are composed of cells that randomly inactivate the same X chromosome, and 3) that clonal expansion can occur in normal squamous epithelium. In fact, skewed X inactivation in a subset of normal polyclonal tissues is an expected sequela of early developmental events. X inactivation occurs in each cell of female 46, XX embryos at about the time of implantation.[27] The choice of which X (paternally or maternally derived) chromosome is inactivated in each cell is random, but in the peri-implantation embryo, the pool of stem cells for most future adult tissues is quite small, on the order of 10 (skeletal muscle and lymph node) to 19 (blood lymphocytes) stem cells for most tissues, with 15 cells calculated for skin.[28] Individuals with a constitutive imbalance of X inactivation in polyclonal tissues may reflect the chance inactivation of the same parental allele in a majority of these few stem cells. If all skewing in normal epithelium is based on truly random events, one would expect approximately equivalent frequencies of skewing toward the high and low molecular weight alleles. However, in eight of nine skewed normal tissues in our series, the low molecular weight allele was preferentially amplified after *Hha*I digestion. This raises the possibility that technical artifact contributed to these results, as biased amplification of the lower molecular weight HUMARA allele during PCR has been described in suboptimal PCR conditions, including those of salt contamination or DNA damage.[22] The fact that we saw no biased amplification of undigested DNAs from normal and lesional specimens indicates that the manipulations of digestion are a potential source of bias. Irrespective of the possibility of bias, the effect on result interpretation is minimized when lesion clonality is inferred by comparison of PCR results between lesional DNA and matched normal tissue controls.

The second observation of interest was the identification of monoclonal hyperplasias. Moreover, this phenomenon was observed in lichen sclerosis, suggesting further that clonal expansion could precede the neoplastic phenotype. In one case, skewing characteristic of a clonal population was observed in both lichen sclerosis and hyperplasia from the same case (Figure 2). A monoclonal pattern was also observed in a hyperplasia adjacent to a VIN, although it is conceivable that the two represent a continuum of the same process (Figure 1). Although this assay may not resolve the question of whether the monoclonal patterns are relevant to the risk of cancer in these cases studied, it does indicate that clonal expansion may occur in the absence of, and possibly precede, the morphological atypia characteristic of VIN. Moreover, these observations are consistent with those recently reported in the skin and oral mucosa. Recent studies of cutaneous squamous epithelium have suggested that clonal expansion may occur in response to ultraviolet irradiation, representing

an important event in the pathogenesis of squamous cell carcinoma that precedes the invasive process.[29] A recent report examining loss of heterozygosity in a spectrum of squamous lesions from the head and neck ranging from hyperplasia to cancer identified loss of heterozygosity in 31% of histologically benign squamous hyperplasias and nearly all of squamous dysplasias and carcinomas *in situ*.[30] The authors proposed a model for head and neck tumorigenesis in which progressive genetic alterations (allelic loss) accompany the evolution of squamous neoplasia. In this scenario, allelic loss in some benign-appearing hyperplasias identifies these alterations as potential precursor lesions.[30] Clonal studies of vulvar squamous epithelium provide the opportunity to test a similar pathway in the genesis of vulvar neoplasia.

Certain aspects of the development of vulvar squamous cell carcinomas suggest that genetic alterations occur in the squamous epithelium preceding development of a histological carcinoma: 1) these tumors occur in older women, irrespective of the presence or absence of human papillomaviruses, 2) they often follow lichen sclerosis and vulvar hyperplasias, and 3) vulvar squamous atypia is frequently found adjacent to the carcinomas.[31] Our results indicate that not only vulvar intraepithelial neoplasia but also vulvar hyperplasias and lichen sclerosis merit further study as potential precursors to invasive vulvar cancer.

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