Chromosome 17 Aneusomy Detected by Fluorescence *in Situ* Hybridization in Vulvar Squamous Cell Carcinomas and Synchronous Vulvar Skin

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Vulvar squamous cell carcinoma (SCC) affects a spectrum of women with granulomatous vulvar diseases, human papillomavirus (HPV) infections, and chronic inflammatory vulvar dermatoses. To determine whether there is evidence of chromosomal instability occurring in synchronous skin surrounding vulvar SCCs, we investigated abnormalities in chromosome 17 copy number. Samples of SCC, vulvar intraepithelial neoplasia (VIN), and surrounding vulvar skin were obtained from all vulvar excisions performed for squamous neoplasia at Albany Medical College from 1996 to 1997. Histological categorization, fluorescent *in situ* hybridization (FISH) for the α satellite region of chromosome 17, DNA content by image analysis, and Ki-67 labeling were evaluated. Controls of normal vulvar skin not associated with cancer were used for comparison. One hundred ten specimens were obtained from 33 patients with either SCC or VIN 3 and consisted of 49 neoplastic, 52 nonneoplastic, and 9 histologically normal vulvar skin samples. The majority of SCCs (88%) and a minority (18%) of VIN 3 excisions were associated with lichen sclerosis. Normal vulvar skin controls did not exhibit chromosome 17 polysomy (cells with more than four FISH signals), whereas 56% of normal vulvar skin associated with cancer did. Moreover, the frequency of polysomy significantly increased as the histological classification progressed from normal to inflammatory to neoplastic lesions. The largest mean value and variance for chromosome 17 copy number was identified in SCCs (2.4 ± 1.0) with intermediate values identified, in decreasing order, for SCC *in situ* (2.1 ± 1.0), VIN 2 (2.1 ± 0.8), lichen sclerosis (2.0 ± 0.5), lichen simplex chronicus (1.9 ± 0.4), and normal skin associated with SCC (1.8 ± 0.4) compared with control vul-

var skin (1.5 ± 0.05). Concordance of chromosome 17 aneusomy between cancers and synchronous skin lesions was found in 48% of patients. Loss of chromosome 17 was identified 5% of all samples and was significantly associated with women with SCC *in situ* (HPV-related). Both DNA content and Ki-67 labeling positively and significantly correlated with mean chromosome 17 copy number ($r = 0.1$, $P = 0.007$). A high degree of genetic instability (aneuploidy) occurs in the skin surrounding vulvar carcinomas. As these events could be detected in histologically normal skin and inflammatory lesions (lichen sclerosis), chromosomal abnormalities may be a driving force in the early stages of carcinogenesis. Differences in chromosomal patterns (loss or gain) support the concept of at least two pathways in vulvar carcinogenesis. (*Am J Pathol* 2000, 157:973-983)

Vulvar squamous cell carcinoma (SCC) is an uncommon cancer that afflicts a spectrum of women with granulomatous vulvar diseases, human papillomavirus (HPV) infections, and chronic inflammatory vulvar dermatoses.¹⁻³ Histopathological, molecular, and epidemiological studies have revealed two subsets of vulvar SCCs: 1) younger women with HPV risk factors and 2) older women without HPV risk factors who are commonly affected by vulvar lichen sclerosis (LS). It is suspected that the pathway to both human papillomavirus-positive and -negative vulvar SCCs may involve not only obvious precancerous changes, eg, VIN 3 (SCC *in situ*), but also biological events in the vulvar mucosa that precede the onset of morphological atypia.^{1,2}

Carcinogenesis is believed to be a multistep process driven by an accumulation of genetic defects that directly affect genes regulating cell birth and cell death.⁴ An underlying genetic instability is argued to be the basis for the multiple mutations found in most cancers.⁵ There is

Supported by a Dermatology Foundation Research Award Grant (1999-2000).

Accepted for publication June 14, 2000.

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now evidence that this genomic instability occurs at two distinct levels: the nucleotide level and the chromosome level.⁶ Losses or gains of whole or large portions of chromosomes in tumor cell populations (aneuploidy) characterize chromosomal instability and are found in most human tumors.⁷ Recently, Duesberg et al⁸ demonstrated that aneuploidy is proportional to the degree of genetic instability in cell lines and hypothesized that aneuploidy is an important mechanism of altering and simultaneously destabilizing normal cellular phenotypes leading to the karyotypic and phenotypic heterogeneity of cancer cells. In the case of head and neck SCCs, increasing aneuploidy (measured as either chromosome polysomy or DNA aneuploidy) can be followed through a progressive multistep histological pathway,^{9–11} supporting this theory. In the case of vulvar SCC and its precursor lesions, there are few karyotypic or DNA content analyses to document whether chromosomal aberrations are significant in its genesis.^{2,12–20}

To determine whether chromosomal abnormalities occur in vulvar cancers and their surrounding skin, we used fluorescent *in situ* (FISH) methods to visualize abnormalities in chromosome 17 copy number in a prospective study of vulvar SCCs. Chromosome 17 was chosen as the object of this investigation because the *p53* tumor suppressor gene, located on 17p, has been implicated in the development of many vulvar SCCs,^{21–28} chromosome 17 polysomy is frequent in some forms of SCC,^{9,11,29–31} loss of chromosome 17 can be found in cervical and cutaneous SCCs,^{32,33} and a susceptibility locus for oncogenic HPV infection has been identified on 17qter.³⁴ In addition to FISH investigation, DNA content and Ki-67 labeling index analyses were performed to determine whether the copy number of chromosome 17 correlated with DNA content aneuploidy (ie, gross chromosomal instability) and growth fraction, respectively.

Materials and Methods

Sample Selection

Fresh tissue samples were obtained from 33 consecutive cases of vulvar surgery for SCC or high-grade vulvar intraepithelial neoplasia (VIN 3) excised at Albany Medical Center from 1996 through 1997. Based on gross examination, samples of vulvar carcinomas, condylomata, vulvar dermatoses, and normal skin (or surgical margins in the absence of grossly normal skin) were collected. These samples were fixed in 10% standard buffered formalin, processed routinely, and stained with hematoxylin and eosin for histological classification. For control specimens, four normal vulvar skin samples were taken from noncancer vulvar biopsies or simple excisions (ie, melanocytic nevi and follicular cysts). The Institutional Review Board for Human Research at Albany Medical College approved this study. Clinical follow-up was obtained for all cases.

Histological Classification

SCCs were classified into three categories as previously described³⁵: typical SCC, basaloid carcinoma, and warty carcinoma. SCC *in situ* (SCCIS)/VIN 3 was classified as either VIN 3 basaloid or warty types (undifferentiated) or VIN 3, differentiated type (carcinoma simplex), based on criteria from the International Society for the Study of Vulvar Diseases.^{36,37} Throughout this text, VIN 3, undifferentiated type (basaloid and warty variants), is referred to as SCCIS because it is morphologically distinct from VIN 3, differentiated type, and is histologically similar to cutaneous SCC *in situ* (Bowen's disease). Diagnosis of specific vulvar dermatoses (eg, lichen simplex chronicus, lichen planus, LS, or spongiotic dermatitides—allergic or irritant contact dermatitis, seborrheic dermatitis) was based on criteria published by Ackerman et al.³⁸ Histological samples without features of the above entities were classified as normal. In addition, SCCs were classified according to histological alterations in the synchronous adjacent vulvar skin, eg, SCCs associated with LS or those associated with SCCIS (HPV-related lesions).

FISH Analysis for Chromosome 17 Copy Number

Unstained paraffin-embedded tissue sections 4 μ m thick were applied to silanized slides and processed with the Oncor chromosome *in situ* hybridization system using the chromosome 17 α -satellite (D17Z1) probe (Oncor, Gaithersburg, MD). Slides were processed with a Ventana Gen II automated hybridization instrument (Ventana Medical Systems, Tucson, AZ). After deparaffinization in xylene and transfer through two changes of 100% ethanol and subsequent rinsing, the slides were placed on the Gen machine. The slides were incubated in 30% Oncor pretreatment solution at 45°C for 30 minutes and Oncor protein digestion solution at 45°C for 45 minutes. The slides were then dehydrated. Oncor unique-sequence, digoxigenin-labeled chromosome 17 probe was prewarmed for 5 minutes at 37°C before manual application. The amount of probe hybridization mixture was approximated according to the size of the target (10 ml probe mixture per 22 $\times$ 22 mm tissue area). Denaturation was accomplished at 69°C for 5 minutes before the slides were incubated overnight at 37°C. After the overnight hybridization and three posthybridization stringency washes, fluorescein-labeled, anti-digoxigenin detection reagent was manually applied for 28 minutes at 37°C. After removal of the slides from the instrument, each was counterstained with 18 μ l of propidium iodide/antifade (1:2) and covered with a coverslip.

The FISH nuclear signals were counted in 40 nuclei from two to three separate fields (80–120 cells counted *in toto*) under $\times$ 100 magnification, using an oil immersion objective on a fluorescent microscope (Zeiss Corporation, Thornwood, NY). Counting was performed using criteria proposed by Hopman et al.³⁹ Specifically, only distinct isolated nuclei were counted, fluorescent signals were scored as true hybridization events only if they were

approximately the same size and intensity as those in adjacent cells, and paired signals were scored as single events. Regions of preparations with background fluorescence or without hybridization signals were not scored, and cells that did not exhibit a signal were not counted. These cells without a signal were mostly truncated nuclei (or cells with loss of chromosome(s) 17) and consisted of less than 5% of all cells in scanned fields. A chromosome 17 copy number index was calculated by dividing the total number of fluorescent chromosome 17 signals by the number of cell nuclei counted. We defined aneusomy for chromosome 17 as an index range that was less than 1.4 or greater than 1.7—a range three standard deviations away from the calculated mean index found for control normal skin samples in this study (see Table 2). A normalized chromosome index was determined by dividing the chromosome 17 index of the study samples by the mean found for the normal vulvar skin controls and was used to graphically compare histological subsets (see Figures 1, 3, and 4). To determine whether minor subpopulations of aneusomic cells were present, we used the thresholds published by Southern and Herrington,⁴⁰ who documented that the sensitivity for the detection of clones of cells exhibiting tri- and tetrasomic populations is 17–18% and 10–11%, respectively, of the total population of cells counted.

DNA Content Analysis

Sections were cut at 5 μ m and deparaffinized in xylene and ethanol. Slides were immersed in 5 N HCl for 60 minutes and stained for 60 minutes, using the Feulgen stain provided by the Cell Analysis Systems (CAS) (Lombard, IL) staining kit. Slides were then rinsed, treated with acid alcohol, dehydrated, and cleared.

Keratinocytes, neoplastic and nonneoplastic, were analyzed using the CAS 200 image analysis system and Quantitative DNA Analysis Plus (QDA+) software program. A minimum of 100 nonoverlapping nuclei were chosen for analysis. A DNA content index for each sample was derived from the mean DNA content of the G₀/G₁ compartment of the analyzed tumor or nonneoplastic keratinocytes divided by the DNA content of the G₀/G₁,

Figure 1. Normalized chromosome 17 indices for control vulvar skins and all histological classifications of cancer-associated vulvar skin. The controls exhibit little variance, in marked contrast to vulvar skin associated with squamous cell carcinoma. Note the apparent increase in both variance (copy number heterogeneity) and mean copy number index in the progression from cancer-associated normal skin to nonneoplastic inflammatory dermatitis to squamous neoplasia. Loss of chromosome 17 (monosomy) was identified in known non-LS, HPV-related lesions. The mean chromosome 17 copy numbers are normalized to those found in control vulvar skin. Box-and-whisker plots of chromosome 17 copy number index are listed by histological categorization: ls, lichen sclerosis; lsc, lichen simplex chronicus (a.k.a. squamous hyperplasia); scca, squamous cell carcinoma; sccis, squamous cell carcinoma *in situ*; n, normal adjacent vulvar skin; vin, vulvar intraepithelial neoplasia. The line in the middle of the box represents the median. The box extends from the 25th percentile to the 75th percentile (interquartile range). The whiskers mark upper and lower adjacent values. Circles represent outliers. The y axis represents the normalized chromosome 17 index (study group/control group).

compartment of lymphocytes with known diploid DNA content processed similarly. Samples were classified as (peri-) diploid or aneuploid, based on visual inspection of the histogram (if peaks were identifiable outside the diploid or tetraploid range) or if the DNA content index equaled or exceeded 1.23.

Immunohistochemistry

Using the Ventana ES automated diaminobenzidine (DAB) immunohistochemical system (Ventana Medical Systems, Tucson, AZ), expression of antibodies to Ki-67

Table 1. Summary of Histological Findings in 110 Samples from 33 Consecutive Vulvar Excisions

Association	Squamous cell carcinoma (71 samples/16 excisions)			Vulvar intraepithelial neoplasia 3 (39 samples/17 excisions)	
	SCCIS (6/1)	Lichen sclerosis (61/14)	Other (4/1)	SCCIS (27/14)	VIN 3, differentiated (12/3)
Histological diagnosis					
SCC	2	18	1	—	—
SCCIS	3	0	0	16	0
VIN 3, differentiated type	0	0	0	0	3
VIN 2	0	1	0	4	1
Condyloma	0	0	0	2	0
Lichen sclerosis	0	32	0	0	7
Lichen simplex chronicus	0	4	0	5	1
Granulomatous vulvitis	0	0	1	0	0
Normal skin	1	6	2	0	0

SCCIS, squamous cell carcinoma *in situ* (synonymous with VIN 3, basaloid or warty types).

Table 2. Chromosome 17 FISH Signal Distribution, Index and Frequency of Aneusomy, DNA Content Frequency of Aneuploidy, and Ki-67 Labeling Index for 110 Samples from 33 Vulvar Excisions for Invasive Squamous Cell Carcinoma or VIN 3 (Squamous Cell Carcinoma *in situ*)

Histological diagnosis		1 signal*	2 signals	3 signals	4 signals	5+ signals
Controls, normal vulvar skin	<i>n</i> = 4	44% (34–51%)	54% (45–65%)	2% (0–4)	0.3% (0–1%)	0
Squamous cell carcinoma	<i>n</i> = 21	31% (0–61%)	40% (6–64%)	8% (0–21%)	6% (0–19%)	15% (0–19%)
Squamous cell carcinoma <i>in situ</i>	<i>n</i> = 19	41% (0–75%)	37% (2–61%)	5% (0–21%)	2% (0–14%)	13% (0–95%)
VIN 3, differentiated type	<i>n</i> = 3	26% (18–30%)	55% (50–65%)	8% (4–11%)	5% (1–11%)	5% (0–16%)
VIN 2	<i>n</i> = 6	41% (17–69%)	35% (22–65%)	8% (8–19%)	5% (0–14%)	12% (0–40%)
Lichen sclerosis	<i>n</i> = 39	37% (15–59%)	44% (20–46%)	7% (1–19%)	4% (2–9%)	12% (0–45%)
Lichen simplex chronicus	<i>n</i> = 10	41% (15–70%)	43% (24–61%)	7% (2–11%)	3% (0–8%)	8% (0–42%)
Normal skin, study population	<i>n</i> = 9	42% (11–67%)	46% (29–67%)	6% (1–15%)	2% (0–8%)	3% (0–18%)
Other histologies						
Condyloma (no. 2)		34/39%	51%	14/5%	1/4%	0/1%
Granulomatous dermatitis		58%	42%	0	0	0

Squamous cell carcinoma *in situ* is also known as VIN 3, undifferentiated type.

*Mean percentage of cells showing one FISH signal per cell (range).

†No. of signals/cells counted $\pm$ standard deviation (range).

‡Aneusomic if 17 index is >1.7 or <1.4 and aneuploid if DNA content index >1.23 or exhibited an abnormal histogram.

§Ki-67 labeling index determined as the number of cells labeled per 100 basal keratinocytes.

(Mib-1) (Ventana Medical Systems; prediluted) per 100 basal keratinocytes was measured and cited as the Ki-67 labeling index.

Statistics

Statistical analysis was carried out with STATA software (College Station, TX). Differences between groups were tested by the χ^2 test for dichotomous variables, the *t*-test for continuous variables with equal variance, and the Mann-Whitney *U*-test for continuous variables with unequal variance. Linear regression analysis was used to determine correlations between study variables. Analysis of equality of variance was determined using Bartlett's test across categories. Survival analysis was tested by using the Cox proportional hazards model. For those patients with SCC arising in the setting of LS or SCCIS (HPV-related lesions), a proposed stepwise histological model of neoplastic progression was tested by linear regression methods. Specifically, the presence of a linear relationship was evaluated for the proposed sequence of 1) normal skin-lichen simplex chronicus (squamous cell hyperplasia)-lichen sclerosis-VIN-SCC or 2) normal-lichen simplex chronicus-condyloma and VIN 1/2-SCCIS (VIN 3)-SCC, testing for the factors evaluated above. Lichen simplex chronicus (squamous cell hyperplasia) is included in both schemata because of its frequent occurrence adjacent to vulvar SCC (this study)^{2,41} and its suspected role in vulvar carcinogenesis.⁴² The criterion for significance for all tests was $P \leq 0.05$.

Results

Clinical Findings

One hundred ten samples were collected from vulvar excisions from 33 women with a mean age of 65 years (range 26–90). These surgical excisions were performed for SCC (*n* = 16 (48%)) or VIN 3, all types (*n* = 17 (52%)). Invasive SCC patients were significantly older than VIN 3 patients (72 years *versus* 56 years, $P = 0.001$, *t*-test). Four invasive SCCs (23%) were second primary SCCs (the original SCC was completely excised, and the second SCCs developed 1 year or more after the first SCC). Metastatic SCC was present in the lymph nodes of 10 vulvar SCC patients (59%) at the time of surgery. Two of these cases were from second primary SCCs. Eight (47%) SCC patients had died of disease at the end of follow-up (mean 26, median 25, range 2–125 months). None of the SCCIS patients have had a local recurrence or evidence of progression to invasive SCC to date.

Histological Findings

Sample collection varied per patient, based on the size of the surgical excisions and the presence of clinically identifiable abnormalities in the skin surrounding the tumor. On average, three samples were collected per patient/surgical specimen (range 1–7). See Table 1 for a complete list of histological diagnoses identified in the 110 samples collected and subcategorized by histological association. Seventy-one samples (63%) were collected from the SCC patients. Fifteen of these SCCs were of the

Table 2. *Continued*

Chromosome 17 signal index [†]	Aneusomy [‡]	DNA content index	Aneuploidy [‡]	Ki-67 index [¶]
1.55 ± 0.05 (1.5–1.6)	0	1.07 ± 0.08 (0.97–1.19)	0	20 ± 10% (10–32%)
2.4 ± 1 (0–77%)	76% (1.4–5.2)	1.68 ± 0.58 (0.86–2.77)	71%	53 ± 22% (22–91%)
2.1 ± 1.0 (1.2–4.9)	79%	1.51 ± 0.38 (0.77–2.47)	84%	66 ± 30% (17–146%)
2.1 ± 0.4 (1.8–2.5)	100%	1.70 ± 0.34 (1.34–2.01)	100%	44 ± 30 (17–76)
2.1 ± 0.8 (1.2–3.2)	83%	1.69 ± 0.75 (1.27–3.2)	100%	51 ± 57% (12–162%)
2.0 ± 0.5 (1.5–3.4)	59%	1.22 ± 0.35 (0.54–2.3)	44%	23 ± 18% (2–95)
1.9 ± 0.4 (1.5–2.8)	70%	1.13 ± 0.35 (0.74–1.76)	30%	16 ± 11% (7–40)
1.8 ± 0.4 (1.3–2.6)	56%	1.16 ± 0.22 (0.94–1.46)	33%	13 ± 9% (5–29%)
1.8/1.8 1.4	100% 0	0.9/1.28 1.06	50% 0	11%/32% 19%

conventional keratinizing type, and one was a warty type (associated with warty-type SCCIS). The majority (15 (88%)) of invasive SCCs were associated with LS, and all showed conventional morphology. The remaining two SCCs were associated with warty-type SCCIS in one case and granulomatous vulvitis in the adjacent skin in the second case. Thirty-nine samples (37%) were collected from vulvar excisions for VIN 3. Three of these patients (12%) had VIN 3, differentiated type (a.k.a. carcinoma simplex), with LS in the adjacent skin, and 14 (88%) had SCCIS (VIN 3, undifferentiated type) with either a basaloid morphology (two patients (12%)) or warty morphology (12 patients (76%)).

Fluorescent in Situ Hybridization for Chromosome 17 Results

See Table 2 for the chromosome 17 FISH signal distribution per nucleus, mean copy number index, and frequency of aneusomy by histological classification of vulvar skin samples.

Demonstration of Chromosome 17 FISH Signal Number Heterogeneity in the Skin Surrounding Vulvar Squamous Neoplasia and Squamous Cell Carcinomas

Normal control vulvar skin (non-cancer-associated) showed mostly one or two signals per nucleus, with less than 4% of cells in three of four controls exhibiting three signals per cell and 1% of cells in one of four controls showing four signals. The mean chromosome 17 index for these normal control skins was 1.55 ± 0.05 signals per cell. In contrast, normal skin associated with carcinomas had a higher mean chromosome 17 index of 1.8 ± 0.4 and a larger and unequal variance compared with controls ($P = 0.007$, Bartlett's test for equal variance). However, this elevated chromosome 17 index was not significantly different compared with controls ($P = 0.07$,

Mann-Whitney *U*-test). Similarly, comparing all other histological classifications to normal controls showed significantly larger and unequal variances as well as significant differences in the mean chromosome 17 index (all $P = 0.04$, Mann-Whitney *U*-test) for some of the histological categories. For SCC and SCCIS samples, the variance and mean index of chromosome 17 copy number were greatest and significantly larger than those of all other histological classifications, except for VIN 2/3 differentiated lesions ($P = 0.04$). Figure 1 graphically illustrates the marked heterogeneity in mean chromosome 17 FISH signals for vulvar skin affected by squamous neoplasia by histological categorization, in contrast to control vulvar skin not associated with carcinoma.

In general, keratinocytes from VIN 2/3, SCCIS, and SCC excisions, regardless of histological classification, exhibited two or more signals more frequently than controls (21% versus 2% cells with more than two signals; $P = 0.0001$, Mann-Whitney *U*-test). Moreover, the frequency of two or more chromosome 17 copies was greatest for SCCs and lowest for normal skin in excision specimens (30% versus 11%; $P = 0.01$, Mann-Whitney *U*-test), with all other values by histological classification falling within this wide range.

Chromosome 17 Aneusomy Is Frequent in Vulvar Skin Affected by Carcinoma

Aneusomy for chromosome 17 (if 17 FISH signal index >1.7 or <1.4 ; controls' mean index 1.7 ± 3 standard deviations) was found in 70% of all samples studied. For neoplastic lesions (SCC, SCCIS, and VIN 2/3), the presence of chromosome 17 aneusomy was significantly more frequent than for nonneoplastic lesions (79% versus 62%, $P = 0.04$, χ^2 test), with the highest frequency identified in VIN 2/3 lesions (89%). Monosomy for chromosome 17 (assumed if index $1.7 < 1.4$) was identified in six specimens (5%): one SCC, three SCCIS, one lichen sim-

plex chronicus, and one normal skin from five patients. Polysomy of chromosome 17 (any sample with cells showing five or more signals per cell) were identified in 73 (66%) of study samples: 15 (71%) SCC, 12 (63%) SCCIS, 5 (56%) VIN 2/3, 28 (54%) LS, 7 (70%) lichen simplex chronicus, 1 condyloma (50%), and 5 (56%) normal specimens from 30 patients. No control vulvar skin specimen contained cells with more than four signals. In addition, cells with more than four signals were not identified in five of the six specimens with chromosome 17 monosomy ($P = 0.01$, χ^2 test). The one exception to this observation was a SCCIS sample (patient 8; see Table 3) with two adjacent cells containing more than five signals per cell. It is worth noting that 30% of cells from a sample of lichen simplex chronicus from this same patient exhibited five or more FISH signals per cell.

Southern and Herrington⁴⁰ have documented that the sensitivity for the detection of minor subpopulations of cells exhibiting tri- and tetrasomic populations is 17–18% and 10–11%, respectively, of the total population of cells counted. With the use of these thresholds, subpopulations of keratinocytes exhibiting trisomy 17 (if >18% of cells counted contained three signals) was found in nine specimens (8%): four SCC, four LS, and one VIN samples from seven patients. Tetrasomy 17 cell populations (if >11% cells counted had four signals) were identified in nine specimens: four SCC, one SCCIS, three LS, and one VIN sample from seven patients. Three samples (two SCC and one LS) derived from two patients contained distinct populations of cells showing trisomy 17 or tetrasomy 17.

Concordance of Chromosome 17 Aneusomy in Adjacent Vulvar Skin and Matched Squamous Cell Carcinomas

Fourteen (48%) of 29 patients with multiple vulvar samples exhibited aneusomy for chromosome 17 in both the primary lesion (eg, SCC or VIN 3) and surrounding non-neoplastic skin. In most cases, this concordance of aneusomy was concomitant to a relatively similar mean chromosome 17 FISH signal index (see Table 3 for a list of patients). There were three exceptions to this observation (patients 8, 18, and 31). Two cases showed a polysomy in the nonneoplastic skin that was associated with loss of chromosome 17 (monosomy 17) in the carcinoma (patients 8 and 18). A third case exhibited an increase in one chromosome 17 copy from LS to VIN 3, differentiated type (patient 31). Two patients had subpopulations of cells with trisomy 17 (patients 22 and 30) in both their SCC and adjacent LS. Similarly, for tetrasomy 17 (patients 17 and 30), both SCC and adjacent LS specimens contained tetrasomic cell populations.

In three patients, aneusomy of chromosome 17 was identified in the synchronous skin, but not in the corresponding SCC or SCCIS. Two of these patients had LS-associated SCCs in which elevated chromosome 17 indices were present in one LS specimen (chromosome 17 index 1.9) from one patient and in two lichen simplex chronicus samples (1.7 and 2.0) and a VIN 2 lesion (1.8) from the other case. The LS sample and one of the two

Table 3. List of Patients Exhibiting Concordance for Aneusomy between Synchronous Skin and Adjacent Squamous Carcinoma or Carcinoma *in Situ*

Patient	Histology	Chromosome 17 copy number index	DNA content index
1	SCC	2.1	2.3
	LS	1.8	1.4
2	SCC	3.0	2.6
	LS	3.4	1.4
4	SCC	5.2	1.8
	LS	4.6	1.4
5	SCC	1.7	1.7
	LS	2.1	1.4
8	SCCIS	1.2	2.5
	VIN 2	1.2	1.4
	LSC	2.8	1.2
11	SCCIS	4.1	1.7
	VIN 2	3.2	1.7
14	SCC	3.8	2.2
	SCC	3.0	2.1
	SCC	3.0	2.8
	LS	3.4	1.5
17	SCC	1.9	1.91
	LS	2	1.07
	N	1.9	1.46
18	SCC, warty type	1.39	1.50
	SCC, warty type	1.48	0.9
	SCCIS	1.8	1.23
	N	1.9	1.38
22	SCC	2.4	1.24
	LS	2.4	0.78
	LS	3.2	0.54
30	SCC	2.7	1.8
	LS	2.0	1.06
	LS	2.7	1.09
	N	2.6	1.34
31	VIN 3, differentiated	2.5	1.67
	VIN 3, differentiated	2.1	2.01
	VIN 2	2.9	1.35
	LS	1.9	1.76
32	SCCIS	1.8	1.91
	LSC	2.2	1.02
33	SCC	1.9	1.80
	LSC	1.8	1.02

LS, lichen sclerosis; LSC, lichen simplex chronicus (a.k.a. squamous hyperplasia); SCC, squamous cell carcinoma; SCCIS, squamous cell carcinoma; N, normal adjacent vulvar skin; VIN, vulvar intraepithelial neoplasia.

lichen simplex chronicus specimens contained cells with a chromosome 17 polysomy (≥ 5 FISH signals per cell). Both SCCs had a chromosome 17 index of 1.6 and did not contain any cells showing polysomy. In the third case, one of two VIN 2 lesions had a chromosome 17 index of 1.8 and cells exhibiting chromosome 17 polysomy, whereas the other VIN 2 lesion and SCCIS had indices of 1.6 and 1.5, respectively, and no polysomic cells.

Correlation of Chromosome 17 Signal Index with DNA Content and Ki-67 Labeling Indices

See Table 2 for mean DNA content index, frequency of DNA content aneuploidy, and Ki-67 labeling index by histological classification.

To test whether the increasing copy number of chromosome 17 correlates with increasing DNA content

and/or increasing number of cells in the active phases of the cell cycle, DNA content by the Feulgen method and the percentage of keratinocytes labeled by Ki-67 were measured. Ki-67 (MIB-1) is a cell cycle antigen expressed throughout all active stages of the cell cycle (G_1 , S, G_2 , and M).⁴³ For both analyses, significant positive (but very weak linear) covariances were identified with increasing chromosome 17 copy number index (DNA content index $r = 0.1$, $P = 0.004$, and Ki-67 labeling $r = 0.05$, $P = 0.02$). In addition, increasing Ki-67 labeling weakly correlated with increasing DNA content ($r = 0.05$, $P = 0.01$).

Abnormalities of FISH signals for pericentromeric α satellite repeat probes (ie, D17Z1) are thought to represent aneuploidy and not increased cell cycling (proliferation), as centromeric DNA does not separate until mitosis.⁴⁴ Theoretically, increasing percentages of three and four FISH signal counts in this study could represent cells in the G_2 phase before chromosome condensation or early mitotic cells. If this is the case, then a significant positive covariance should be identified when the Ki-67 index is compared to the frequency of three and four FISH signals per cell. In fact, only increasing percentages of two and five or more FISH signals per cell significantly correlated with increasing Ki-67 index. Positive covariances were found for both signal distributions, with the strongest correlation identified for two FISH signals per cell ($r = 0.1$) compared with that for five signals or more ($r = 0.07$) ($P = 0.002$, linear regression analysis).

In accord with the differences identified for the chromosome 17 signal index, significant differences were identified for both means and variances for measurements of DNA content and Ki-67 labeling indices across histological classifications. The variance for DNA content, but not the Ki-67 labeling index of control vulvar skin, was significantly lower than that of histological normal skin associated with vulvar carcinomas ($P = 0.03$, Bartlett's test for equality of variance). However, the mean values for DNA content or Ki-67 indices were not significantly different ($P > 0.1$, Mann-Whitney U -test). Comparing SCC and SCCIS to normal vulvar skin, lichen simplex chronicus, and LS showed significantly larger and unequal variances as well as significant differences in both the mean DNA content and Ki-67 labeling indices (all $P = 0.04$). No differences for either DNA content or Ki-67 labeling indices were identified when we compared SCC and SCCIS with VIN lesions ($P = 0.04$). LS lesions showed both significantly larger variance and larger mean values for both DNA content and Ki-67 labeling indices compared with those for normal vulvar skin from carcinoma excisions (all $P = 0.04$, Bartlett's test for equality of variance and Mann-Whitney U -test).

Gross Genetic Aberrations Are Frequent and Widespread in Vulvar Skin Affected by Carcinoma

No control normal vulvar skins exhibited DNA aneuploidy. However, 60% of all vulvar skin samples from both SCC and VIN excisions were found to contain an aneuploid DNA content, with the highest frequency seen in VIN lesions and the lowest percentage in lichen simplex

chronicus specimens. Eighty-five percent of all vulvar specimen ($n = 94$) had an abnormal number of chromosome 17 copies and/or an abnormal DNA content. Of the 16 specimens (15%) with neither aberrant finding, 11 were nonneoplastic (eight LS, one LSC, and two N) and five were neoplastic (four SCC and one SCCIS). Forty-five percent ($n = 50$) of all specimens evaluated showed both DNA aneuploidy and chromosome 17 aneusomy. If only neoplastic lesions were considered ($n = 49$), there was a significant correlation of chromosome 17 aneusomy with DNA aneuploidy, with 70% exhibiting both and 10% exhibiting neither ($P = 0.004$, χ^2 test). However, for nonneoplastic lesions a significant correlation was not identified—24% showed both aneusomy and aneuploidy, 18% had neither, and 68% showed one or the other ($P = 0.6$ χ^2 test).

Evidence for Increased Genetic Alterations during Multistep Carcinogenesis in the Setting of Vulvar Lichen Sclerosus

Vulvar SCCs can be divided into two distinct subsets: 1) SCCs associated with HPV-related risk factors and precursor lesions, eg, SCCIS (VIN 3, undifferentiated type), or 2) SCCs associated with LS.^{1,2} If the development of SCC of the vulva is a multistep process resulting from an accumulation of genetic changes, we would expect to observe an increase in the frequency of chromosome aneusomy with histological progression from histologically normal skin to carcinoma. In the setting of vulvar LS, the proposed histological stepwise model of normal-lichen simplex chronicus-LS-VIN-SCC significantly correlates with evidence of gross genetic alterations as measured in this study. Significant positive correlations were identified for progressive histological stages with increasing chromosome 17 copy number index ($r = 0.1$, $P = 0.01$ linear regression), DNA content index ($r = 0.1$, $P = 0.0001$ linear regression), and Ki-67 index ($r = 0.3$, $P = 0.0001$ linear regression). By multivariate linear regression analysis, both the Ki-67 index and the DNA index were independent variables correlating with histological stepwise progression ($P = 0.02$). Figure 2 depicts examples of increased FISH chromosome 17 signals in both LS and SCC. Figure 3 graphically depicts the upward trend of increasing copy number 17 through progressive histological stages toward SCC in the setting of LS.

For squamous neoplasia arising in the setting of SCCIS (HPV-related),^{26,35,45-47} a similar histological schema was used: normal-lichen simplex chronicus-condyloma and VIN 2-VIN 3-SCC (Figure 4). In this proposed pathway of carcinogenesis, no correlation for chromosome 17 copy number index ($r = -0.03$, $P = 0.9$ linear regression), a weak positive correlation for DNA content ($r = 0.1$, $P = 0.02$), and a significant positive correlation for Ki-67 index ($r = 0.3$, $P = 0.001$) were identified with advancing histological stage. By multivariate linear regression analysis, Ki-67 was found to be an independent predictor of advancing stage ($P = 0.003$). In addition, monosomy 17 occurred significantly more often in patients with SCCIS (HPV-related lesions) compared with

Figure 2. Aneusomy of chromosome 17 in LS and SCC is common. **A:** LS with keratinocytes exhibiting more than two signals per cell. **B:** Invasive SCCs demonstrated the highest number and greatest frequency of high chromosome 17 copy number. Note that many of the cells contain more than three signals per cell.

patients with LS (5/32 (16%) versus 1/75 (1%), $P = 0.002$, χ^2 test). The LS-associated sample with monosomy 17 was found in a normal skin sample adjacent to SCC.

Correlation with Clinicopathological Parameters

The presence of lymph node metastases was the only factor in this study that correlated with survival (80% dead with lymph node metastases versus 29% dead without lymph node metastasis, $P = 0.02$, Cox proportional hazards model). The presence of aneusomy, DNA aneuploidy, or elevated Ki-67 index did not influence survival in this study group or correlate with the presence of lymph node metastases of SCC (all $P > 0.3$). Five (56%) patients alive without disease had SCCs with chromosome 17 aneusomy, compared with seven (88%) patients who were dead from metastatic SCC.

Discussion

Neoplastic diseases have been defined as proliferative disorders characterized by uncoordinated cell growth and are believed to be the result of the progressive accumulation of mutations, via a multistep pathway, in the genes that directly control cell birth and cell death.^{6,48} In this study, the correlation of increasing Ki-67 index (growth fraction) with histological stepwise model(s) of

Figure 3. Multistep histological model of carcinogenesis in the setting of vulvar LS. Normalized chromosome 17 index reveals a significant upward trend ($r = 0.1$, $P = 0.001$ linear regression) toward increasing polysomy from histologically normal skin to SCC arising from a background of LS. The data were derived from 73 samples from 17 women with vulvar LS that developed SCC¹⁵ or VIN 3, differentiated type.² Box-and-whisker plots of normalized chromosome 17 copy number are listed by histological categorization: normal adjacent vulvar skin (1), lichen simplex chronicus (2), lichen sclerosus (3), VIN (4), and SCC (5). The line in the middle of the box represents the median. The box extends from the 25th percentile to the 75th percentile (interquartile range). The whiskers mark upper and lower adjacent values. Circles represent outliers. The y axis represents the normalized chromosome 17 index (study group/control group).

vulvar carcinogenesis clearly highlights the importance of cell proliferation in both the development and progression of vulvar SCC. Correspondingly, both DNA content aneuploidy and chromosome 17 aneusomy coexisted with this increasing growth fraction, attesting to the interrelation between cell-cycle control and genomic stability.

Lengauer et al^{6,49} have suggested that either a mutated mismatch repair gene or a chromosome segre-

Figure 4. Multistep histological model of carcinogenesis in the setting of HPV-related lesions (VIN 3 undifferentiated type/squamous cell carcinoma *in situ* (SCCIS)). Normalized chromosome 17 index reveals a downward trend, loss of chromosome 17 ($r = -0.03$, $P = 0.9$ linear regression), from SCCIS to squamous cell carcinoma arising in association with SCCIS. The data were derived from 33 samples from 15 women with SCCIS, one of which developed invasive SCC (two samples). Box-and-whisker plots of normalized chromosome 17 copy number are listed by histological categorization: normal adjacent vulvar skin (1), lichen simplex chronicus (2), condyloma and VIN 2 (3), SCCIS (4), and SCC (5). The line in the middle of the box represents the median. The box extends from the 25th percentile to the 75th percentile (interquartile range). The whiskers mark upper and lower adjacent values. Circles represent outliers. The y axis represents the normalized chromosome 17 index (study group/control group).

gation gene could act as a mutator gene, driving the carcinogenic process toward cancer. Of these two mechanisms, the putative chromosome segregation gene defects are thought to be more common and have a dominant phenotypic effect. In support of this theory is the fact that most human malignancies exhibit alterations of chromosome number, ie, gain or loss of whole or large portions of chromosomes.⁷ Moreover, a single cancer can show heterogeneous karyotype, indicating the generation of new chromosomal variations and underlying chromosomal instability.⁷ The exact genes involved in chromosomal instability are unknown, but defects in genes that control cell-cycle checkpoints are suspected.⁵⁰ An alternative theory is that genetic instability of cancer cells is caused by aneuploidy itself.^{8,51} Once cells are aneuploid, they will continue to be subject to asymmetrical segregation every time they divide—a process termed “chromosome error propagation.”⁵² Our demonstration of a significant increase in the frequency of chromosome 17 polysomy through the histological pathway of normal to nonneoplastic disorders to VIN to SCC and the significantly higher copy number of chromosome 17 found in SCC support the concept of carcinogenesis via a dominant chromosomal instability phenotype.

The results reported here for aneuploidy in normal and nonneoplastic skin samples are based on individuals who had a 100% risk of developing cancer. Thus the aneuploidy identified in these samples may reflect a predisposition of these individuals to tumor formation (so-called mutator phenotype)⁵ and would not necessarily be secondary to a concomitant process such as LS. Nonetheless, there is evidence of aneuploidy occurring in vulvar skin affected by LS not associated with vulvar carcinoma.^{2,53} In addition, women affected with vulvar SCC are at high risk for second primary SCCs.^{2,54,55} These observations suggest that the theory of “field cancerization” applies equally to vulvar skin and to aerodigestive epithelium.^{56,57} This theory implies that the vulvar epithelium has been subjected to mutagens (eg, oncogenic HPVs and/or free radicals from persistent inflammation)^{1,2,58} and has been initiated and therefore is at risk for one or more cancers. Confirmation of widespread genetic damage to vulvar skin is evidenced by the frequent presence of DNA content aneuploidy and chromosome 17 aneusomy in both normal and inflammatory (nonneoplastic) skin.

Flowers et al⁵⁹ recently reported a greater number of molecular alterations, particularly allelic losses at 3p, in HPV-negative vulvar SCCs, in contrast to HPV positive SCCs. In this study, the gain of chromosome 17 in LS-associated SCCs and loss of chromosome 17 in HPV-related SCC further support the current concept of two pathways for vulvar carcinogenesis at the chromosomal level in addition to epidemiological, histological, and molecular dichotomies.^{1,2} Vulvar SCC arising in the setting of LS may well represent an example of inflammation-promoted carcinogenesis.^{2,60} Similar to LS-associated vulvar SCC, bladder SCC associated with schistosomiasis⁶¹ contains numerous chromosomal aberrations, including gains of chromosome 17 that are distinct from

those aberrations identified for non-schistosomiasis-related transitional cell carcinoma.⁶² In support of the possibility that loss of chromosome 17 is an important step in HPV-related vulvar SCCs is the documentation of chromosome 17 monosomy in CIN III.⁶³ The majority of cervical SCCs are HPV related (commonly HPV 16).⁶⁴ Furthermore, HPV oncoproteins E6, E7, and E5, as well as the viral regulatory protein E2 of the high-risk HPVs (eg, HPV-16 or HPV-18), are known to influence and override the different checkpoints of the cell cycle.^{65–70} Whereas the E7 and E2 proteins influence the G₁/S phase checkpoints more, E6 has dysregulatory effects on multiple mitotic checkpoints, ie, G₁/S, S/M, and G₂ spindle assembly checkpoints. Disruption of one or more cell cycle checkpoints, resulting in mitotic nondisjunction and/or DNA rereplication, may be the means by which high-risk HPVs induce genomic instability, manifested as numerical and structural chromosomal abnormalities.

A high degree of genetic instability (aneuploidy) occurs in the skin surrounding vulvar carcinomas. As these events could be detected in histologically normal skin and inflammatory lesions (lichen sclerosus), chromosomal abnormalities may be a driving force in the early stages of carcinogenesis. Differences in chromosomal patterns (loss or gain) support the concept of two pathways in vulvar carcinogenesis.

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