Molecular Biology of Squamous Cell Carcinoma of the Anus: A Comparison of HIV-Positive and HIV-Negative Patients

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The molecular mechanisms involved in progression of squamous cell carcinoma of the anus (SCCA) are poorly elucidated, as well as the potential role of HIV infection. Loss of heterozygosity (LOH) is one of the mechanisms responsible for inactivation of tumor suppressor genes. We hypothesized that HIV-induced immunosuppression may contribute to an alternate molecular pathway in SCCA progression, through persistence of human papillomavirus infection within the anal canal. This study was undertaken to compare the molecular biology of SCCA in HIV-positive (HIV+) and HIV-negative (HIV−) patients. We retrieved tumor specimens from 18 HIV− and 10 HIV+ patients diagnosed with SCCA in two institutions. DNA from tumor and normal tissues was extracted and then amplified by polymerase chain reaction. LOH was investigated at 14 loci: three at 18q (*DCC*), two at 13q (*Rb*), three at 17p (*p53*), three at 11q, one at 2p, and two at 5q (*APC*). LOH was defined by a tumor DNA-to-normal tissue DNA ratio of >2. HIV+ patients were younger (36 ± 7 years versus 53 ± 13 years, $P = 0.001$) and showed a trend toward tumors of larger size (3.7 ± 1.6 cm versus 2.6 ± 1.5 cm, $P = 0.09$). The median $CD4^+$ count in HIV+ patients at the time of diagnosis was 74×10^6/L (range, 5−900). The overall frequency of LOH was 17.3% (41 LOH of 236 informative loci). Tumors in HIV− patients were more likely to present LOH than were tumors in HIV+ patients (24.1% versus 6.6%, $P = 0.0004$). Differences between the two groups with regard to allelic losses were also observed at specific loci, such as 18q (41% [HIV−] versus 0% [HIV+], $P = 0.05$), 17p (43% versus 10%, $P = 0.09$), and 5q (33% versus 0%, $P = 0.12$). Consistent LOH on chromosomes 17p, 18q, 5q, and 11q were observed in HIV− patients with SCCA. By contrast, allelic losses at 17p, 5q, and 18q seem to be rare in tumors of HIV+ individuals. These data suggest that immunosuppression may promote SCCA progression through an alternate pathway and that persistence of HPV infection within the anal canal may play a central role in this process. (J GASTROINTEST SURG 2004;8:1024–1031)

KEY WORDS: Anal cancer, biology, HIV, genes, HPV, loss of heterozygosity

Squamous cell carcinoma of the anus (SCCA) includes 1.5% of all digestive system cancers in the United States, with 4,000 new cases and 500 deaths in 2003.[1] SCCA is considered a sexually transmitted disease that is clinically related to infection with high-risk human papillomaviruses (HPVs).[2,3] The best-characterized factor in the molecular biology of SCCA is the integration of HPV types 16/18 DNA into anal canal cell chromosomes.[4,5] However, in an experimental model of neoplasm, the presence of high-risk HPV was not sufficient to induce transformation and tumor progression.[6] Studies on squamous cell carcinomas of the cervix have demonstrated that in addition to HPV integration, the neoplastic process requires the loss of tumor suppressor gene (TSG) function.[7] Thus, HPV infection is thought to be a necessary factor but insufficient on its own to promote malignant transformation in SCCA.

Since 1990, numerous epidemiologic studies from the United States have demonstrated a high incidence

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of anal cancer among AIDS patients.[8] Although most immunocompetent individuals experience spontaneous regression of anogenital HPV infection, HIV-positive (HIV+) patients are more likely to have persistent HPV infection than are HIV-negative (HIV−) patients.[9] In the case of male homosexuals, it has been possible to investigate the effects of HIV-mediated immune suppression on HPV infection, as dual infection with both viruses is relatively common in this population.[10] Although the mechanisms of the HPV–HIV interaction are poorly understood, it has been suggested that HIV-related immune suppression is responsible for an enhanced expression of HPV infection in the anal canal, which may lead to HPV-induced epithelial abnormality.[11]

Persistence of high-risk HPV within the anal canal of immunosuppressed individuals has important biological implications, because high-risk HPV (mostly types 16 and 18) encode for three oncoproteins with growth-stimulating and transforming properties (E5, E6, and E7).[12] The E6 protein contributes to cell transformation and the development of malignancy through the binding and inactivation of p53 protein, an important negative regulator of cell growth.[13] It has also been demonstrated that the binding of E7 to retinoblastoma (Rb) protein results in degradation of this protein via ubiquination.[14] Unlike many cancers in which LOH at 17p is demonstrated in 40–60% of cases, the *p53* gene is frequently wild-type in HPV-related cervical cancers.[15] Thus, the notion has arisen that E6-mediated abrogation of p53 protein–dependent apoptosis is equivalent to inactivating mutation of the *p53* gene.[16]

We hypothesized that, first, HIV-induced immune suppression may promote SCCA progression through an alternate molecular pathway and, second, that LOH at various loci harboring known TSGs may not be a necessary step in the progression of SCCA in HIV+ patients due to the persistence of HPV infection in this population. Thus, the aim of this study was two-fold: to identify specific patterns of LOH involved in SCCA progression and to correlate these molecular alterations with HIV status.

METHODS

Patients

Through a preliminary database search, a total of 124 patients who received treatment for SCCA at Mayo Clinic Rochester between 1980 and 2000 were identified. The following parameters were assessed for entry in a computerized database: patient demographics, tumor stage, tumor location (anal canal or anal margin), HIV status, history of anal warts, modalities of treatment including toxicity and side effects, response to chemoradiation therapy, and clinical outcome. A pathologist reviewed all specimens for confirmation of the initial diagnosis and to delineate the area of normal and tumor tissues on specimen. The tumor percentage had to be 70% or greater for our analysis parameters. All patients had given prior approval to have their specimen used for research. The Institutional Review Board approved the study protocol.

Tumor Microdissection and DNA Extraction

Genomic analyses were performed and interpreted by individuals who were blinded to patients' HIV status. A total of 18 specimen from HIV− patients (all from the Mayo Clinic) were deemed appropriate for DNA analysis. Tumor specimens originating from 10 HIV+ patients were retrieved from the Department of Pathology at the University of South California in Los Angeles. The tissues were routinely collected from the endoscopy suite or from the operating theatre, fixed in buffered formalin, embedded in paraffin, and stored for a variable number of months before selection for analysis. Paraffin-embedded blocks were cut with a microtome into 5-μm-thick sections and affixed to glass microscope slides. Using a sterile scalpel blade, areas of normal (nontumor) and cancer tissue were microdissected under a dissecting microscope using a hematoxylin and eosin–stained section as a guide. Care was taken to prevent admixture of microdissected domains and to ensure that identified tissue were removed in a precise manner. Genomic DNA was then extracted from the microdissected tissue using the QIAamp DNA Mini Kit No. 51306 (Qiagen, Venlo, The Netherlands). The specimens were deparaffinized in xylene, purified with absolute alcohol, and centrifuged at 14,000 rpm. The pellets were dried under reduced pressure in a DNA SpeedVac (Savant, Inc., Farmingdale, NY), resuspended in 15 ml of Genereleaser (BioVentures Inc., Murfreesboro, TN), used according to the manufacturer's protocol, and incubated at 55°C overnight in 200 mg/ml proteinase K (Sigma, St. Louis, MO). The specimens were used directly for polymerase chain reaction (PCR) analysis.

Tumor Loss of Heterozygosity Assays

The following microsatellite primers tightly linked to TSGs were used for LOH analyses: D18S35 (*dcc*) and D18S46 (*DCC/Smad4*); D13S270 and D13S319 (*Rb*); tp53, D17S786, and D17S513 (*p53*); D11S29, D11S4127, and D11S925 (*11q*); D5S346 and D5S421 (*APC*); and D2S123 (*2p*). Each pair was optimized

for efficient amplification. One primer from each pair was labeled with a phosphoramidite dye. Each 15-μL reaction contained 2 μL of genomic DNA, 200 μmol/L dNTPs, 1.33 μmol/L each primer, 0.5 U Ampl*Taq* Gold (PE Biosystems), and 1.5−2.5 mmol/L $MgCl_2$. Reactions were cycled in either a Perkin Elmer (Wellesley, MA) 9600 GeneAmp PCR System or an MJR (Waltham, MA) Tetrad Cycler as follows: 10 minutes at 95°C; then 35 cycles of 30 seconds at 95°C, 30 seconds at 58°C or 55°C, and 30 seconds at 72°C; and then a final 10-minute extension at 72°C. Reactions were held at 5° C until analysis. PCR products were resolved on an ABI (Applied Biosystems, Foster City, CA) 3100 DNA sequencer. Genotypes were analyzed using ABI Genotyper 2.5 software.

Definition of Loss of Heterozygosity

Loss of heterozygosity was assessed by comparing the ratio of the peak heights of each allele between normal and tumor DNA samples (N1/N2*T2/T1). For each marker, ratios of 2 or greater were defined as clear evidence for LOH; ratios of 1.5–2 were interpreted as suggestive evidence for LOH; and ratios of 1.5 or lower were considered as absence of LOH.

The results for each marker were then collected to determine LOH at individual loci according to the following: A normal DNA–to–tumor DNA ratio of 2 or greater at one marker at least was considered a strong indication of LOH for the specific locus. A ratio greater than 1.5 but less than 2 at one marker at least was considered indicative for "indeterminate" LOH at the specific locus. When ratios were less than 1.5 for all informative markers, this was considered a clear indication for no LOH at the specific locus.

Statistical Analysis

Statistical analyses were undertaken by means of the software package STATGRAPH 3.0 software for Windows (Statgraph Software Inc., San Diego, CA). Quantitative data were expressed as mean ± SD or median (range). Group comparisons were made using χ^2 or Fisher's exact test for categorical variables, and Student's *t* test for continuous variables. *P* values less than or equal to a two-sided α level of .05 were considered statistically significant.

RESULTS

A total of 18 tumors from HIV− and 10 tumors from HIV+ patients were available for DNA analysis. The distribution of tumors according to location within the anal margin or the anal canal was similar between groups. HIV+ patients with SCCA were exclusively males and were younger than HIV− patients (36 ± 7 years versus 53 ± 13 years, $P = 0.001$). Eight of 10 of the HIV+ patients had $CD4^+$ counts that were below 200×10^6/L at the time of diagnosis. Seventy-two percent of HIV− and 77% of HIV+ patients were alive without evidence of recurrence at the time of last follow-up. Overall, two thirds of patients were treated initially with radiation therapy or a combination of chemotherapy and radiotherapy. However, DNA analysis was performed on pretreatment biopsies to avoid any potential bias due to the effect of adjuvant treatment. The clinical characteristics of patients included in the study are summarized in Table 1.

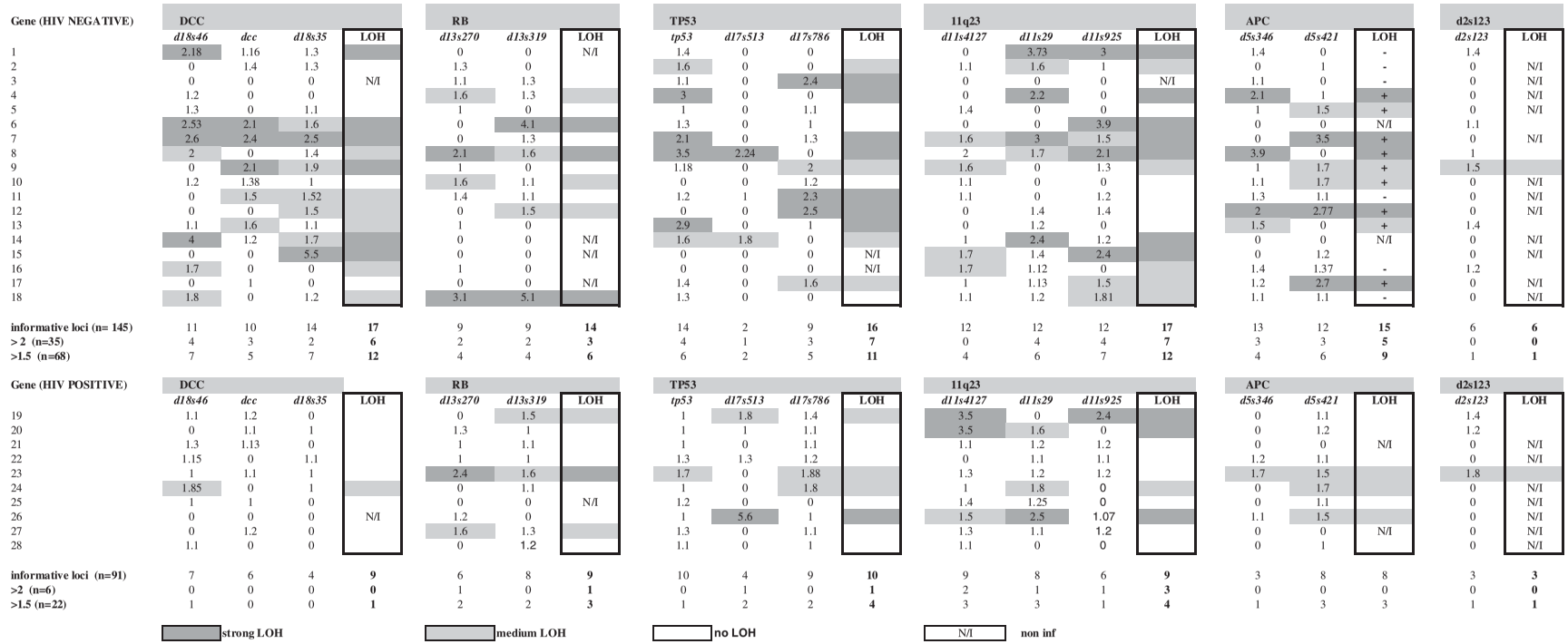
Loss of Heterozygosity at Individual Loci

Of 14 loci tested, 57.5% (145 of 252) were considered informative for LOH in HIV− patients, and 65% (91 of 140) were informative in HIV+ patients. A total of 41 instances of LOH (ratio ⩾2) were observed in the entire group: 35 in HIV− and 6 in HIV+ patients. Thus, when considering all informative loci, SCCAs in HIV− patients were more likely to present with LOH than were tumors in HIV+ patients (35 LOH/145 loci [24.1%] versus 6 LOH/91 loci [6.6%], $P = 0.0004$). When considering a ratio greater than 1.5 for the definition of LOH, a total number of

Table 1. Clinical characteristics of patients with SCCA

Parameter	HIV negative	HIV positive	*P**
Gender (M/F)	5/13	10/0	<0.001
Age (mean ± SD yr)	53 ± 13	36 ± 17	<0.001
Tumor location			0.67
Anal canal	14	7	
Anal margin	4	3	
Tumor size (mean ± SD cm)	2.6 ± 1.5	3.7 ± 1.6	0.09
$CD4^+$ count (median [range])	—	74 (5–900)	
Follow-up (median mo [range])	47 (1–440)	29 (3–67)	0.26
Radiation therapy			1.00
Yes	13	6†	
No	5	3	
Status at last follow-up			1.00
Alive without recurrence	13	7†	
Alive with recurrence	0	1	
Dead	5	1	

*Fisher's exact test or Student's *t*-test, when indicated.
†One patient lost to follow-up.



Gene (HIV NEGATIVE)	DCC				RB			TP53				11q23				APC			d2s123	
	d18s46	*dcc*	*d18s35*	LOH	*d13s270*	*d13s319*	LOH	*tp53*	*d17s513*	*d17s786*	LOH	*d11s4127*	*d11s29*	*d11s925*	LOH	*d5s346*	*d5s421*	LOH	*d2s123*	LOH
1	2.18	1.16	1.3		0	0	N/I	1.4	0	0		0	3.73	3		1.4	0	-	1.4	
2	0	1.4	1.3		1.3	0		1.6	0	0		1.1	1.6	1		0	1	-	0	N/I
3	0	0	0	N/I	1.1	1.3		1.1	0	2.4		0	0	0	N/I	1.1	0	-	0	N/I
4	1.2	0	0		1.6	1.3		3	0	0		0	2.2	0		2.1	1	+	0	N/I
5	1.3	0	1.1		1	0		1	0	1.1		1.4	0	0		1	1.5	+	0	N/I
6	2.53	2.1	1.6		0	4.1		1.3	0	1		0	0	3.9		0	0	N/I	1.1	
7	2.6	2.4	2.5		0	1.3		2.1	0	1.3		1.6	3	1.5		0	3.5	+	0	N/I
8	2	0	1.4		2.1	1.6		3.5	2.24	0		2	1.7	2.1		3.9	0	+	1	
9	0	2.1	1.9		1	0		1.18	0	2		1.6	0	1.3		1	1.7	+	1.5	
10	1.2	1.38	1		1.6	1.1		0	0	1.2		1.1	0	0		1.1	1.7	+	0	N/I
11	0	1.5	1.52		1.4	1.1		1.2	1	2.3		1.1	0	1.2		1.3	1.1	-	0	N/I
12	0	0	1.5		0	1.5		0	0	2.5		0	1.4	1.4		2	2.77	+	0	N/I
13	1.1	1.6	1.1		1	0		2.9	0	1		0	1.2	0		1.5	0	+	1.4	
14	4	1.2	1.7		0	0	N/I	1.6	1.8	0		1	2.4	1.2		0	0	N/I	0	N/I
15	0	0	5.5		0	0	N/I	0	0	0	N/I	1.7	1.4	2.4		0	1.2		0	N/I
16	1.7	0	0		1	0		0	0	0	N/I	1.7	1.12	0		1.4	1.37	-	1.2	
17	0	1	0		0	0	N/I	1.4	0	1.6		1	1.13	1.5		1.2	2.7	+	0	N/I
18	1.8	0	1.2		3.1	5.1		1.3	0	0		1.1	1.2	1.81		1.1	1.1	-	0	N/I
informative loci (n= 145)	11	10	14	**17**	9	9	**14**	14	2	9	**16**	12	12	12	**17**	13	12	**15**	6	**6**
> 2 (n=35)	4	3	2	**6**	2	2	**3**	4	1	3	**7**	0	4	4	**7**	3	3	**5**	0	**0**
>1.5 (n=68)	7	5	7	**12**	4	4	**6**	6	2	5	**11**	4	6	7	**12**	4	6	**9**	1	**1**

Gene (HIV POSITIVE)	DCC				RB			TP53				11q23				APC			d2s123	
	d18s46	*dcc*	*d18s35*	LOH	*d13s270*	*d13s319*	LOH	*tp53*	*d17s513*	*d17s786*	LOH	*d11s4127*	*d11s29*	*d11s925*	LOH	*d5s346*	*d5s421*	LOH	*d2s123*	LOH
19	1.1	1.2	0		0	1.5		1	1.8	1.4		3.5	0	2.4		0	1.1		1.4	
20	0	1.1	1		1.3	1		1	1	1.1		3.5	1.6	0		0	1.2		1.2	
21	1.3	1.13	0		1	1.1		1	0	1.1		1.1	1.2	1.2		0	0	N/I	0	N/I
22	1.15	0	1.1		1	1		1.3	1.3	1.2		0	1.1	1.1		1.2	1.1		0	N/I
23	1	1.1	1		2.4	1.6		1.7	0	1.88		1.3	1.2	1.2		1.7	1.5		1.8	
24	1.85	0	1		0	1.1		1	0	1.8		1	1.8	0		0	1.7		0	N/I
25	1	1	0		0	0	N/I	1.2	0	0		1.4	1.25	0		0	1.1		0	N/I
26	0	0	0	N/I	1.2	0		1	5.6	1		1.5	2.5	1.07		1.1	1.5		0	N/I
27	0	1.2	0		1.6	1.3		1.3	0	1.1		1.3	1.1	1.2		0	0	N/I	0	N/I
28	1.1	0	0		0	1.2		1.1	0	1		1.1	0	0		0	1		0	N/I
informative loci (n=91)	7	6	4	**9**	6	8	**9**	10	4	9	**10**	9	8	6	**9**	3	8	8	3	**3**
>2 (n=6)	0	0	0	**0**	1	0	**1**	0	1	0	**1**	2	1	1	**3**	0	0	0	0	**0**
>1.5 (n=22)	1	0	0	**1**	2	2	**3**	1	2	2	**4**	3	3	1	**4**	1	3	3	1	**1**

Fig. 1. DNA ratios and loss of heterozygosity.

90 instances of allelic imbalance were observed. The differences between groups, however, remained statistically significant for all loci (HIV−; 68 LOH/145 loci [46.9%] versus HIV+; 22 LOH/91 loci [24.1%], $P = 0.0005$). The detailed data of DNA analysis for each marker are summarized in Figures 1 and 2.

When looking at specific TSGs, there were again significant differences between groups for *DCC* (9 instances of LOH of 35 informative loci [25.7%] in the HIV− group versus no instances of LOH of 17 informative loci in the HIV+ group, $P = 0.02$) and for *p53* (8 instances of LOH of 25 informative loci [32%] in the HIV− group versus 1 instance of LOH of 23 informative loci in the HIV+ group, $P = 0.02$). By contrast, detailed DNA analysis for each individual marker failed to reveal any difference at 11q23 (8 instances of LOH of 36 informative loci [22.2%] in the HIV− group versus 4 instances of LOH of 23 informative loci [17.3%] in the HIV+ group, $P = 0.74$).

Loss of Heterozygosity at Specific Loci

Again, LOH was the most consistent between the two groups at 11q23. A total of 10 instances (38.5%) of LOH at that site were identified: 7 (41%) in the HIV− group versus 3 (33%) in the HIV+ group. The differences in allelic imbalance between groups were the most obvious on chromosomes 18q (41% [HIV−] versus 0% [HIV+], $P = 0.05$), 17p (43% versus 10%, $P = 0.09$), and 5p (33% versus 0%, $P = 0.12$). The differential distribution of chromosomal aberrations between groups is detailed in Figure 3.

Using a cutoff value of 1.5, differences between groups for LOH at specific loci remained significant for *DCC* (68.7% [HIV−] versus 30% [HIV+], $P = 0.01$). However, differences between groups were no longer significant at 17p (61% [HIV−] versus 25% [HIV+], $P = 0.10$) and 5q (60% [HIV−] versus 25% [HIV+], $P = 0.19$).

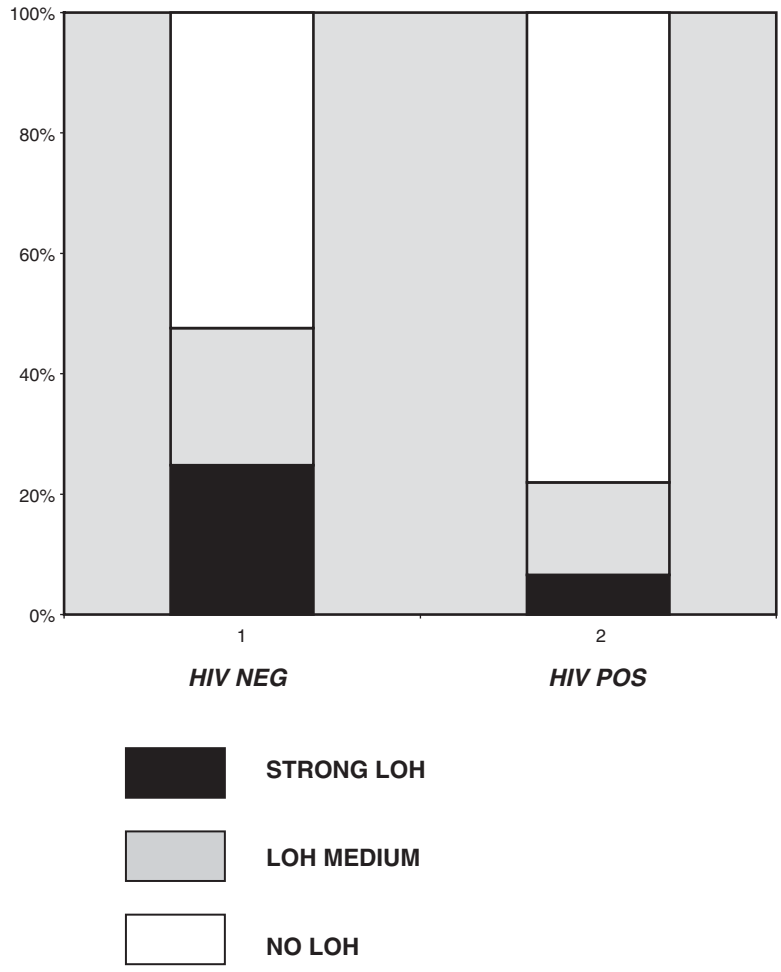


Fig. 2. Percentage of loss of heterozygosity (LOH) for all loci in HIV-negative and HIV-positive patients. *P* value = 0.0004 (strong LOH) and 0.0005 (strong plus medium LOH).

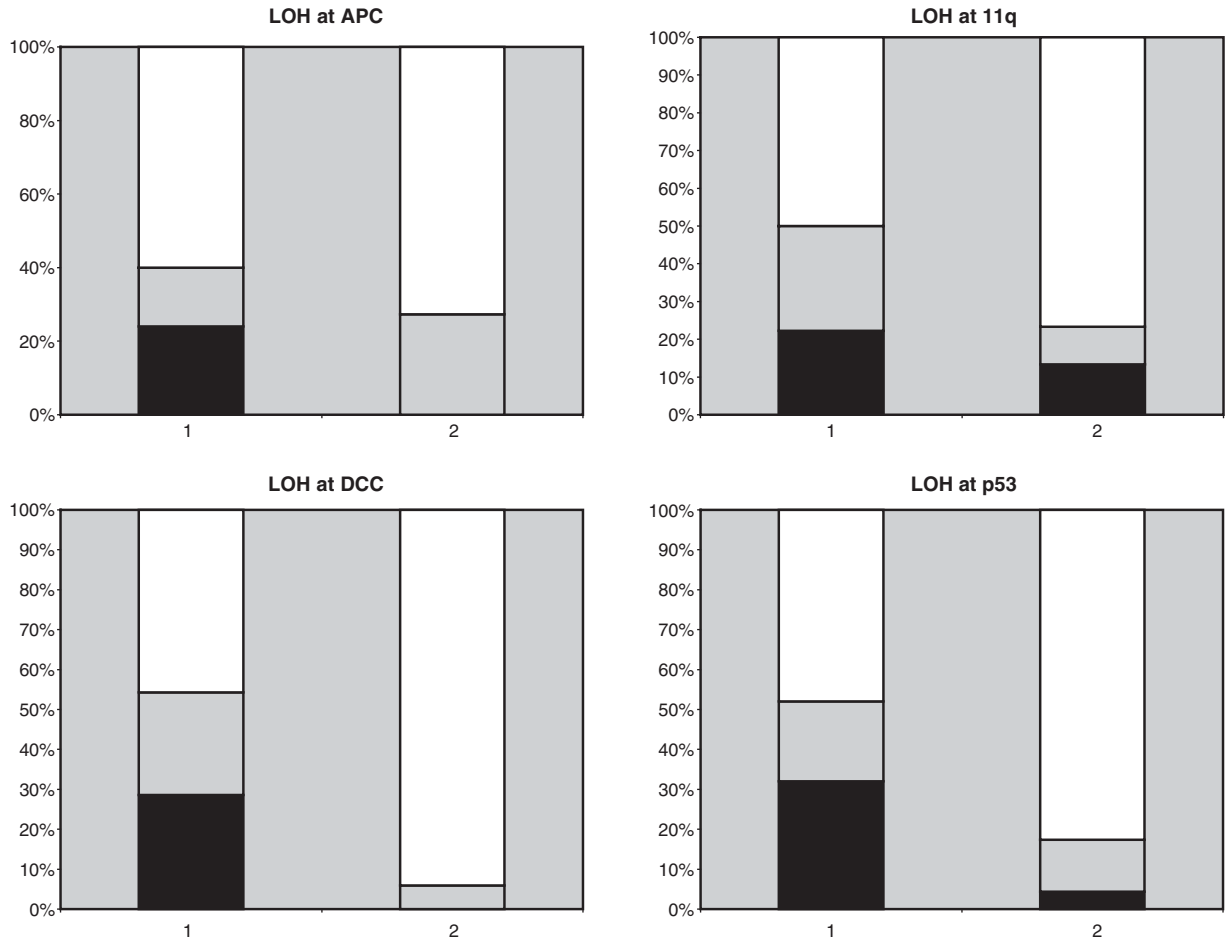


Fig. 3. Comparison of loss of heterozygosity at specific loci between HIV-negative (1) and HIV-positive (2) patients.

DISCUSSION

It is accepted that genetic events, whether induced by or independent of HPV infection, are required for progression of anal cancer. The data presented here indicate that the molecular mechanisms involved in SCCA progression markedly differ between HIV+ and HIV− individuals. Consistent LOH on chromosomes 17p, 18q, 5q, and 11q were observed in HIV− patients with SCCA. By contrast, allelic imbalance in general and, more specifically, LOH at 17p, 5q, and 18q was less likely to occur in tumors of HIV+ individuals. A similar distribution of LOH at 11p was documented in SCCA of HIV+ and HIV− patients.

Although the association between HIV infection and HPV-related anogenital neoplasia has been repeatedly demonstrated through epidemiologic studies, these findings so far have not been substantiated by genetic analysis. In addition, the molecular mechanisms involved in progression of SCCA are poorly elucidated, with relatively few data available.[17,18] In this study, consistent LOH at 11q was documented in 38.5% of SCCA, which is in accordance with a previous series, in which Heselmeyer et al.[19] identified allelic imbalance at 11q in 9 of 23 cases (40%). In fact, the 11q23 region has received much scrutiny, since Hampton et al.[20] demonstrated that 62% of cervical carcinomas had allelic deletions on chromosome 11q. Furthermore, in vitro studies have demonstrated that HPV-mediated immortalization of human keratinocytes requires LOH at 11q and/or 18q.[21] In summary, a TSG on chromosome 11q23 seems to be implicated in carcinogenesis SCCA, and this chromosomal aberration appears to be relatively independent from HIV status.

Our results also indicate that allelic imbalance on chromosomes 17p, 18q, and 5q differ markedly between HIV+ and HIV− patients. In the latter group, LOH (defined by ratio >2) at these loci were documented in 43%, 41%, and 33% of cases, respectively. It is interesting to note that the same TSGs (*p53*, *DCC*, and *APC*, respectively) have been previously implicated in the progression of colorectal cancer, a

neoplasia that is independent of HPV infection.[3,22] Thus, in HIV− patients, the sequence of genetic events seems similar to what is observed in non–HPV-related digestive cancers. An immediate implication is that the necessary accumulation of multiple allelic losses requires a long latency period between the time of HPV infection (peak incidence in the 20s) and the appearance of SCCA in this population (mid-50s).

Our data also indicate that although LOH at 17p and 18q is quite common in immunocompetent individuals, mutations at these loci are rarely observed in HIV-infected patients. First, it is important to note that our patient population was severely immunosuppressed at the time of diagnosis, with $CD4^+$ counts of less than 200×10^6/L in 8 of 10 patients. Previous studies indicate that there is an inverse relationship between $CD4^+$ level and HPV DNA level in the anal canal.[23] It is likely that HIV+ patients in this study had persistent infection with high-risk HPV. HPV-infected cells in the epithelium of the anal canal have the functional equivalent of mutations in *p53* and *Rb*, due to the interaction of their respective proteins with viral oncoproteins E6 and E7.[24] On the basis of our data and the existing literature, we can distinguish three key events in the pathogenesis of SCCA in HIV+ patients: 1) the integration of HPV DNA in the cellular genome; 2) E6-mediated functional inactivation of p53 protein; and 3) LOH on chromosome 11q.

In conclusion, the data presented here demonstrate that *DCC* and *p53* mutations are not required for SCCA progression in HIV+ patients; persistence of HPV infection within the anal canal may play a central role in this process and explain the differences observed with the molecular patterns of these tumors in immunocompetent individuals. In the future, administration of an HPV-16/18 vaccine might help in reducing the incidence of SCCA in HIV-infected patients.[25]

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Discussion

Dr. Robert Beart (Los Angeles, CA): Is it a different mechanism or do you think it is a subset of the other mechanism? Is that mechanism in play in non–HIV-positive patients?

Dr. Gervaz: I think it is the same mechanism, and probably the aim of the mechanism is to inhibit the function of the p53 protein. But this may be achieved either through a mutation of the gene or through an inhibition of the protein, and it is obviously much easier to have an inhibition of the protein than to have a mutation, because it takes much longer.

Dr. Walter Koltun (Hershey, PA): I may have missed it, but did you subtype your HPV infections in each of your patients?

Dr. Gervaz: No, we did no HPV testing in this population. So we have no data regarding which subtypes of HPV were implicated in these tumors.