MIB 1 and p53 in penile intraepithelial and invasive squamous HPV – related lesions

MIB1 e p53 em lesões penianas escamosas intraepiteliais e invasoras; relações com o virus do papiloma humano (HPV)

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Abstract

The aim of this investigation was to study the cell proliferation index (MIB-1) and p53 expression in 24 invasive squamous cell lesions (ISL, CEC), 7 high-grade squamous cell intraepithelial lesions (HSIL) and 11 low-grade intraepithelial lesions(LSIL) of the penis; and correlate them with age, HPV occurrence and the histopathological features. In addition, HPV status was detected by *in situ* hybridization (ISH) in 20 invasive lesions. MIB1 and p53 expressions were evaluated by percentiles and scores (1 to 9, according dispersion of marked nuclei plus reaction intensity) Results: the mean age of occurrence was 62.3 yrs for the ISL; 41.6 yrs for HSIL and 33.2 yrs in the case of LSIL. The MIB-1 showed its highest expression in HSIL (46.1%), followed by the ISL (34.5%), and the LSIL (13.3%). p53 over-expression was mainly seen in 90.9% of LSIL; in 70.9% of ISL and in 57.1% of HSIL The cytohistopathological signals for HPV were seen in 72.7% of low grade lesions, in 87% of the invasive lesion and 100% of the severe dysplasia. The HPV-DNA was detected in 30% of the ISL. In conclusion, MIB-1 and p53 showed to be independent markers and were straight related to the progression and severity of these lesions. The p53 scores increased from the low to the high grade and invasive lesions and probably suggests an early inhibition by HPV proteins, mutations coming up later. There is a clear age-related prevalence of lesions, similar to the vulvar and cervical HPV related-lesions' evolution, but penile carcinoma is delayed.

Key words: squamous cell carcinoma; penile neoplasms; human papillomavirus; in situ hybridization; p53; MIB-1.

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Recebido em dezembro de 2000.

Resumo

O objetivo deste estudo foi investigar o índice de proliferação celular (MIB-1) e a expressão de p53 em 24 carcinomas escamosos invasores (CEC), 7 lesões de alto grau (LIMAG) e 11 de baixo grau (LIMBG) do pênis, segundo a idade. Além do percentual, MIB1 e p53 foram avaliados por escores (baixos, de 1 a 5 e altos, de 6 a 9) segundo o grau de dispersão (1 a 3) de núcleos marcados versus intensidade da reação (1 a 3). A presença do HPV foi estimada pelos sinetes citohistopatológicos em todos os casos e, em 20 CEC, por hibridização in situ com sonda de amplo espectro.

Resultados: a idade média (anos) foi de 62,3 nos CEC, de 41,6 nas LIMAG e 33,2 nas LIMBG. MIB1 foi expresso em 46,1% (LIMAG), 34,5% (CEC) e 13,3% (LIMBG). A p53 mostrou -se expressa nas LIMBG (90,9%), nos CEC (70,9%) e nas LIMAG (57,1%). Todavia, os escores mais altos de MIB1 e de p53 (6 a 9) predominaram nos carcinomas e nas lesões de alto grau. Os sinetes associados ao HPV foram vistos em 100% das LIMAG, 87% dos CEC e 72,7% das LIMBG. O DNA do HPV foi detectado em 30% dos CEC (6/20). Conclusões: a p53 e o MIB1 mostraram-se avaliadores independentes, mais relacionados à gravidade da lesão; não houve relação significativa entre idade, p53 e MIB1. As lesões intraepiteliais ocorreram em faixas etárias semelhantes às descritas para a vulva e o colo uterino; todavia, o carcinoma peniano é mais tardio. Houve nítida associação com os sinetes de HPV porém a HIS mostrou baixa sensibilidade nos CEC (30%). O incremento dos escores de p53 com o grau da lesão sugere que de início há acúmulo por hiperfunção e inibição (pelas proteínas do HPV),as mutações ocorrendo durante a progressão. Já o MIB1 evidenciou que papilomas podem ter escores altos e CEC escores baixos (mormente os verrucosos, de comportamento mais loco-regional)

Palavras-chave: carcinoma de células escamosas; neoplasias penianas; papillomavirus humano; hibridização in situ; p53; MIB-1.

INTRODUCTION

Described since ancient times, the penile carcinoma is one of the most feared human tumors due to its extremely mutilating nature. Geographically heterogenic in distribution, the prevalence of penile carcinoma shows important parallels to the vulvar and cervical cancer. A number of investigations have shown that the two types of malignancy are correlated, revealing high rates of cervical cancer in the partners of individuals with penile neoplasia.[1,2] As in cervical carcinogenesis, the HPV infection is the main cofactor involved in penile cancers, which is also related to early onset of sexual activity and large number of sexual partners.[2,4-6] The penile carcinoma occurs in less than 10 in one million of men, per annum. It has high prevalence in mostly rural areas of South and Central American countries, and in African countries.[2,6-8] Some of the world's high-risk areas for penile cancer are in Brazil, occurring mainly in North (5,3%) and Northeast (5,7%) states. According to the 1991 data from the Brazilian National Cancer Institute, the prevalence rates of this disease in two main cities of the Northeast area of Brazil are 8,3 (Recife) and 5,7 (Fortaleza) per 100.000 men.[8]

The epidemiological importance of penis carcinoma comes from its strong relation to a venereal disease, the HPV infection.[3,5,8,9] American studies indicate HPV infection as the most common sexually transmitted viral disease, where 80% of sexual partners of infected women have anogenital lesions.[10] The papillomavirus is a non-enveloped icosahedron virion of 54 nm diameter, with a pro-

tein coat (capsid) composed of 72 capsomeres connected by bridges at their bases. Its structure shows a circular, double-stranded DNA genome of approximately 8000 base pairs. Due to the related capsid proteins, papillomaviruses are antigenically similar and are not divided into serotypes; instead, they are subdivided into genotypes based on the extent of DNA relatedness.[11] The distinct types of HPVs are classified based on tissue tropism (epitheliotropism), the type of the induced lesion and oncogenic potential. Currently, about 30 types infecting the anogenital tract are known.

The complete genetic evaluation of HPV reveals that coding sequences are distributed in three frames along one strand of DNA. The other strand is blocked by stop signals in all three frames. These frames are three functional regions, called the early and the late regions and a noncoding upstream regulatory region (URR). The products from the early region are responsible for viral replication, transcription control, and cellular transformation. The E6 and E7 encode for critical oncoproteins required for viral replication; as well as host cell immortalyzation and transformation, inactivating p53 and pRb nuclear proteins respectively.[8] Experimental *in vitro* data indicates that the gene product from high oncogenic risk HPV (E6 and E7) play important role in the nuclear protein interaction of p53 and pRb respectively. The expressions of these viral oncoproteins are essential to keratinocyte immortalization; as also to keep the cancerous cell phenotype.[12-14]

The p53 protein is a transcription factor that regulates the cell cycle specially, when the DNA is damaged.[15,16] P53 mutations have rarely been studied in penile carcinoma or in their pre-malignant lesions. In addition to penile intraepithelial neoplasia,[17] the p53 is also detected in papillomas; but only rarely in penile scar or other penile lesions.

The association of mutated p53 with the occurrence of high risk HPV genotype is a common finding in penile as well as in cervical lesions. Lam *et al*.[18] showed, by immunohistochemistry studies, a overexpression of p53 in 17 of 42 penile carcinomas; and in a majority of those cases the HPV DNA was detected, suggesting p53 mutation as an oncogenic risk factor in these tumors.

It is known that the cells in which the p53 is inactivated loose the capacity to arrest the cell cycle in G1 phase or the mechanism of apoptosis, resulting in accumulation of genetic mutations which could eventually lead to malignancy.[11,19,20] Wild-type p53 protein has a short half-life of about 5 to 20 minutes inside the cell nuclei; in contrast the mutated p53 is more stable. Apparently, this change interferes in the degradation mechanism.[21] The p53 mutation is the most frequent genetic change in the different human cancer types.[22-26] It is present in about 37% among all neoplasias and, depending on the anatomic area, it could be reach 7% to 85%. Persons who carry some kind of disability interfering in p53 function will be at higher risks for accumulation of mutations that could eventually transform cells.[20,25,27] p53 can be studied by using direct techniques as PCR and SSCP; or by employing the indirect methods of immunohistochemistry, which uses antibodies to show the overexpression of the altered or mutant protein in the tissues.[17,26]

Recent research has focussed on the relationship between the cellular proliferation and the prognosis of the tumors.[20,21,28-30] The proliferating cells express in their nuclei a nonhistone protein with a short half-life, known as Ki-67. It is in the cell nuclei during the whole cell cycle, except in G0 and early G1 phases.[11] The MIB1 is a new monoclonal antibody that recognizes the epitope of the Ki-67 antigen. It is less sensible to the formalin fixation, and hence is an ideal antibody for retrospective studies.[8,27,31]

The proliferation index is used as a prognostic factor in human malignancies such as mama,[27] esophagus,[32] vulva[33] cancers and most recently in penile verrucous carcinoma;[28] permitting to be applied also for therapeutic purposes.[34]

MATERIALS AND METHODS

Material: forty-two formalin-fixed, paraffin-embedded tissue samples from penile biopsies were retrieved from our files during the period of 1993 to 1999. They were histologically classified according to the Paris-Tolbiac Consensus[35] for squamous (malpighian)

lesions. The samples included 11 low-grade malpighian squamous cell intraepithelial lesions (LSIL), including papillomas; 7 high-grade malpighian squamous cell intraepithelial lesions (HSIL) and 24 invasive squamous carcinomas (ISL).

Method: the immunohistochemistry was performed on 5mm sections mounted on pre-treated slides (silane), using the peroxidase-anti-peroxidase method (PAP). Briefly, sections were deparaffinyzed in xylene, rehydrated, and washed in tris buffer (pH 7.6). Subsequently, they were immersed in citrate buffer (pH 6.0) and heated in a domestic microwave oven at 850w for approximately 10 minutes. The endogenous peroxidase activity was quenched by treating sections with 3% hydrogen peroxide/ methanol solution at room temperature. Immunostaining was performed using p53 DO-7 (Dakopatts, CA, USA) antibody and the monoclonal antibody MIB1 (Immmunotech, Marseille, France) as the primary antibody; both used at 1:50 dilution and incubated overnight at 8ºC in a wet chamber. Rabbit anti-mouse biotinated antibody was used as the secondary antibody, at 1:200 dilution for 30 minutes followed by the ABC complex (Sigma Chemical Co, St Louis, MO, USA) and the 3,3'-diaminobenzidine (DAB - Sigma Chemical Co, St Louis, MO, USA). Counterstainning was performed lightly with Mayer's hematoxylin and coverslips were applied. For in situ hybridization, probes directed against wide spectrum HPV (Dako Corporation, CA, USA), subtypes 6,11,18,30,31,33,35, 45,51 e 52 were used, labeled with biotin and DAB. Specific staining was identified by detecting brown-colored substances in the nuclei. Staining for p53 and MIB1 was evaluated according to the number and distribution of the positively stained cells, by two independent pathologists attributing scores of 1 to 5 (low) or 6 to 9 (high), estimated by the intensity of marking (1 to 3) *versus* unifocal, bi or multifocal dispersion - diffusion (1 to 3) of marked nuclei. Negative control was always performed by primary antibody exclusion. Statistical analyses were performed using Fisher's exact test and the difference is considered significant if p value was ≤ 0.05.

RESULTS

Mild differentiated squamous carcinoma occurred in 45.9% cases; the verrucous in 37.5% and poorly differentiated in 16.6%. The HSIL predominate in the prepuce (57.2%) and in the glans (28.6%) while the warts in the penile skin (55.5%). Almost of all the men with intraepithelial lesions (85%) were examinated because their partner and medical indication, not by themselves free choice.

Related to the age, the invasive neoplasia occurred within the range of 37 to 89 years (average of 62.3); the HSIL from 32 to 51 year (average of 41.6), and the LSIL from 19 to 69 years (average of 33.2). An interestingly observation was the finding of papillomas in aged men. The age significant differences among the groups are shown in Table 1.

The Table 2 shows p53 immunoexpression in the different groups of penile lesions; as well as the results of analyses of correlations between two different lesions and the negative and positive p53 immunoreactions, by the Fisher's exact test. A significant difference among the groups of lesions was also observed; the low-grade lesions often stained for p53 with lower intensity and in less number of cells and basically in deep layers. High scores (6 to 9) predominate in HSIL and ISL but verrucous carcinoma frequently shows low scores.

Table 1. Comparison of squamous cell penile lesions related to age. Fortaleza, Ceará - Brazil

Groups (lesions)	cases	Age average(yrs)	p
ISL x HSIL	24/7	62.3 / 41.6	0,000
ISL x LSIL	24/11	62.3 / 33.3	0,001
HSIL x LSIL	7/11	41.6 / 33.3	0,000

ISL: invasive squamous lesion
LSIL and HSIL: low and high grade intraepithelial squamous lesions

Table 2. p53 immunohistochemical expression and scores in penile squamous cell lesions

Groups	p53+ X p53 -	p	High p53 X low p53	p
ISL x HSIL	17 / 7 X 4 / 3	0.6	13 / 4 X 0 / 4	0.01
ISL x LSIL	17/ 7 X 10 / 1	0.3	13 / 4 X 3 / 7	0.04
HSIL x LSIL	4 / 3 X 10 / 1	0.2	0 / 4 X 3 / 7	0.5

ISL: invasive squamous lesion. LSIL and HSIL: low and high grade intraepithelial squamous lesions Scores: high (6 to 9) and low (1 to 5)

The Figure 1 resume the mainly morphologycal p53 and MIB1 aspects and dispersion of marked nuclei according the severity of the lesion.

There was no significant percentual difference between the low-grade and high-grade

intraepithelial neoplasias, with regard to cellular proliferation index using the MIB1 antibody ; however the high-grade lesions showed higher scores even when compared to the ISL and both to the LSIL (Table 3).

The cytohistomorphological features of HPV were present and frequently associated (2 or more of them) in all of the groups of lesions. In ISL diskeratosis (96.6%) and binucleation (87.5%) were predominant; in HSIL binucleation and coilocytosis occurs in all the cases (7/7) while papilomatosis (100%) and coilocytosis (81.8%) predominate in LSIL.

Of the 24 invasive lesions, 20 of were submitted to ISH studies for DNA HPV presence. The wide spectrum HPV probes demonstrated a 30% positivity (6 of 20 cases), with a diffuse pattern (Figure 1). However, it was not possible to correlate the IHC reactivity to p53, MIB1 or in situ hybridization, as can be seen in Table 4.

In addition, the localization of the HPV-associated lesion in the male genitalia was observed, and it was correlated to histologic diagnosis. The predominant incidence of high-grade lesions occurs in the penile mucosa (prepuce and glans), and the low-grade lesions were prevalent in skin (penile shaft or perineum).

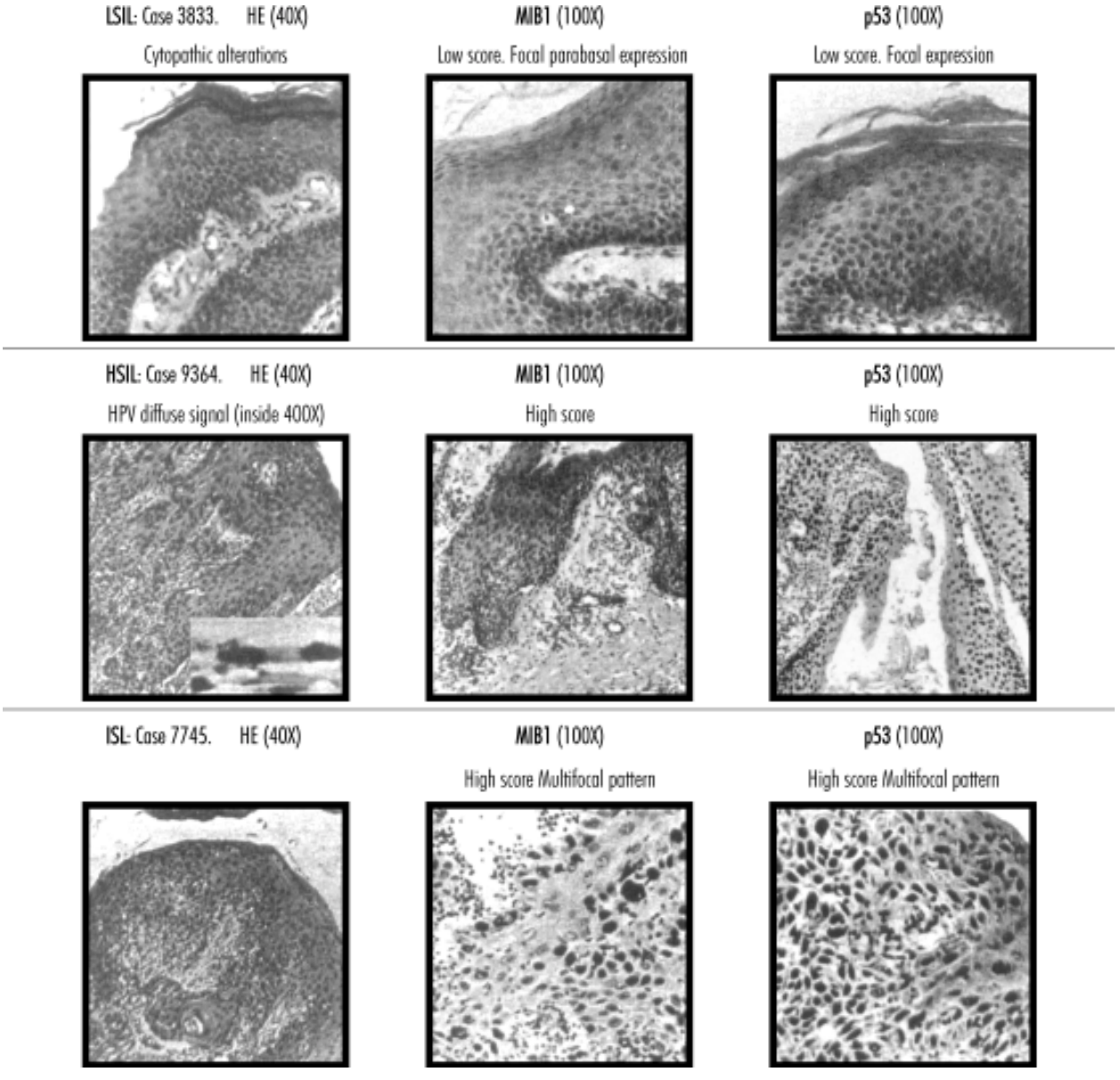

Figure 1. Penile low (LSIL) to high (HSIL) intraepithelial andivasive (ISL) squam ous lesions. Proliferating cellular rate (MIB1), p53 expression and HPV - related signals by cytohistopathologic and hibridization (HIS). Case 3833 - L1: HE, 40X:HPV - related cytopathic alterations. MIB1 (L2) and p53 (L3) low score, focal expression (PAP, 100X) case 9364-H1:HE 40X and HPV-HIS diffuse signal (inside, 400X) MIB.

DISCUSSION

Related to the age, this study demonstrated a significant spectrum of variability for invasive penile carcinoma cases, from 37 to 89 years, with an average of 61 years. Our findings are in accordance with those of Lam *et al.*[18] and Levi *et al.*[23] in which an age variability of 36 to 85 years, with an average of 60 years, was reported. For the high-grade lesions, a range of 32 to 80, with an average of 41.6 years, was observed; which was also close to the average age of 37 years (range 24-50 years) observed by Aynaud *et al.*[36] in Paris. For the low-grade lesions, the average age observed was 33 years (19-69 years).

At the time of the diagnosis, the age of the patients associated with the penile histological lesion type showed a significant difference in the range of the time of evolution of the lesion to penile cancer, compared to what is known in the literature for the carcinogenesis of malpighian lesions. Indeed, the literature sites a time of progression of the lesion of approximately 15 years; where as in our studies, it was observed just at 8 years from low-grade to high-grade lesion.

p53 was expressed in 71% of malignant lesions, showing mainly strong reaction and high scores (6 to 9). Comparing the three lesions histological types studied, related to the IHC of p53, important differences were seen among low to high and invasive squamous cell lesions (p=0.000). Still, our results surpass those of Levi *et al.*[23] who reported 26% of positivity for p53 in penile carcinoma; as also the 50% positivity observed by Castren *et al.*[37] and the 31% and 40% (using antigen retrieval technique) reported by Lam *et al.*[18]

The p53 positivity reaction, related to histologic type of the malignant tumors, was 55% (5 of 9 cases) in verrucose carcinomas, which was a higher value when compared to that of Lam *et al.*[18] (37% of verrucose carcinoma), and was 80% in non-verrucose tumors. The analysis of the results of the high-grade lesions related to p53, showed 75% of positivity. This result agrees well with the 50% of positivity described by Castren *et al.*[37] Tendler *et al.*[30] demonstrated positive immunereaction in every one of four samples of penile papiloma.. Castren *et al.*[37] obtained a 69.2% of IHC reaction using DO-7 and CM-1 antibodies. In 1995, Ranki *et al.*[21] detected 41% of positivity in papillomas using four distinct antibodies: DO-7, CM-1, Pab-240 and DO-1. In our series the immunoreaction positivity was observed in the nuclei and occurred in 90.9% (10 of 11) of the low-grade lesions and papillomas; all of them being focal and in deep layers (low scores), thus suggesting functional instability for p53 also in those type of lesions.

Table 3. MIB1 percentile averages and scores in penile squamous lesions

Groups- MIB1	Averages	Variance	size	p	High/ low scores	p
ISL x HSIL	34,1 / 46,1	460,4 / 541,3	24/7	0.2	9/15 x 7/0	0,006
ISL x LSIL	34,1 / 13,4	460,4 / 30,1	24/11	0.04	9/15 x 1/110	0,08
HSILx LSIL	46,1 / 13,4	541,3 / 30,1	7/11	0.10	7/0 x 1/10	0,0002

ISL: invasive squamous lesion. Scores: high (6 to 9) and low (1 to 5)

LSIL and HSIL: low and high grade intraepithelial squamous lesions

Table 4. ISH (*in situ* hybridization) for HPV, IHC (immunohistochemistry) for p53 and MIB1 in invasive lesions of the penis (ISL). Fisher' exact test

ISH	p53 (+)	p53 (-)	p53 high	p53 low	MIB1 high	MIB1 low
(+)	6	0	3	3	4	2
(-)	11	7	10	1	5	13
	p=0.2		p=1		p=0.3	

ISL: invasive squamous lesion. LSIL and HSIL: low and high grade intraepithelial squamous lesions. Scores: high (6 to 9) and low (1 to 5)

The cellular proliferation index analysis by the MIB1 antibody is frequently used to other types of tumors, such as those of vulva,[33,38,39] esophagus,[32] breast[27] and uterine cervix.[41] Hendricks *et al.*[39] described a 99% of MIB1 focal and diffuse positivity in carcinoma of vulva. In our study, the highest average of IHC positivity was seen in high-grade lesions (46.1%), followed by 34,5% in ISL and 13.4% in papillomas. In papillomas, nuclei labeled in the intermediate layer were observed in three cases, with similar results found also in

high-grade lesions, suggesting aggressivity; possibly transforming some papillomas into high-grade lesions, than the advanced penile dysplasia. As an exception, the verrucose ISL presented low immunoreaction scores for MIB1, due to the keratinization, which is in agreement with the literature and with the unique report in penis.[28]

For ISH, a 30% of positivity to HPV DNA was obtained, in twenty cases of invasive malignant lesions of penis, a substantially lesser result than what was reported earlier in Brazil, in 1986 (51%, according to Villa and Lópes; cited by Levi *et al.*[23]). McCance *et al*.[41] detected a 50.9% of HPV DNA by ISH. Lam *et al*.,[18] studying 42 cases by ISH and PCR, found a value of 30.9% for HPV DNA, which is in agreement with the with the 26% result found in São Paulo, Brazil, by Levi *et al.*[23] Comparing histological classification of tumors and HPV DNA presence for ISH, Gregoire *et al.*[42] found 11% (5 of 45) of verrucose tumors, while we found such tumors in 25% (2 of 8) of our cases.

Finally, there was a significant presence of cytomorphological features of the HPV infection in our material, demonstrating the presence of active infection on the genesis of those lesions.

CONCLUSION

The occurrence and progression of the premalignant lesions followed the same histological and age patterns described for these lesions in other genital sites, but the penile carcinoma seems to occur later. There was no significant correlation among age, p53 and MIB1 in our cases. So, p53 and MIB1 showed to be independent markers, more related to the severity of penial squamous lesions. The overexpression of p53 in the early onset of the intraepithelial squamous lesions, with low to high scores in carcinomas may reflect initial overfunction or inactivation by viral protein and during progression toward malignancy, is probably the result of the ongoing p53 gene mutation. The overexpression of papillomas and the down-regulation of carcinomas, observed in our studies with MIB1 (Ki 67), could be due the high frequency of the verrucous histologic type of these tumors. Even though the HPV histological features are mostly present, just one third of the carcinomas showed HPV- DNA ISH positivity, probably due to the HIS low sensitivity

Finally, this study connected for the first time in a tropical country the penile squamous lesions to oncobiological factors involved in tumor pathogenesis. The next step to follow in this investigation could be the use of more sensitive PCR technique for typing the HPV and associating it to the histological grade of the lesions and to the penioscopy findings; as well as to the p53 IHC expression changes and mutations.

ACKNOWLEDGMENTS

For tecnichal support to Francisco José O. Queiroz, João Carlos da Silva, Ana Cristina S. Lima

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