



# HIGH-RISK PAPILLOMA VIRUS CAPSID PROTEIN (L1) - IMMUNOHISTOCHEMISTRY AND POTENTIAL USE IN CLINICAL PRACTICE

Alves VAF<sup>1</sup>; Wakamatsu A<sup>1</sup>; Santos RTM<sup>1</sup>; Roteli-Martins C<sup>1</sup>; Hilfrich R<sup>2</sup>; Kohlhoff M<sup>2</sup>  
D. Pathology, Inst. Adolfo Lutz, , Brasil<sup>1</sup>; Virofem Diagnostica GmbH, Germany<sup>2</sup>

## INTRODUCTION

Immunohistochemistry has become, in last decades, the most useful adjunct method in surgical pathology, enabling the "in situ" detection of antigens important in the diagnosis of poorly differentiated neoplasia, selection of possible original site for metastatic neoplasia, and, more recently, assessing prognostic or predictive tumor markers. Immunohistochemistry has also proved a very useful tool for the etiological diagnosis of microbial infections in tissue samples, including viruses such as cytomegalovirus<sup>16</sup>, measles<sup>24</sup> and hepatitis B<sup>27</sup>. In this context, immunohistochemical detection of HPV antigens has been searched as a simple tool for identification HPV infection in cellular and tissue samples. Initial work from Jensen et al, 1980 was based on polyclonal serum directed against bovine papillomavirus, which was shown related to L1 proteins of most of the papillomavirus types. The immunoreactivity of such antigens is restricted to highly replicating episomal infections, since integration of viral DNA sequences within the genome of infected cells has been associated to truncation of expression of several viral genes, including those of L1 regions<sup>11,12,23,25,27</sup>. Moreover, HPV segregation in "low-risk" or "high-risk" groups was not achieved by this polyclonal antibody<sup>23</sup>. Afterwards, sporadic reports of the production of monoclonal antibodies to HPV, some of them directed to E6 regions of HPV16 or HPV18, did not achieve diagnostic relevance<sup>22</sup>. Monoclonal antibodies directed to L1 capsid protein of high risk papilloma virus (HR-HPV) were designed for specific immunohistochemical (IHC) detection of HR-HPV infections in intra-epithelial neoplasia<sup>19</sup>. Their benefit and possible clinical inferences have been subject of preliminary reports and still demand further evaluation<sup>6,8,9,15,20</sup>. The aim of the present study is to assess the immunoreactivity of L1 capsid protein of HR-HPV in a series of biopsies of patients representing the spectrum of squamous intra-epithelial lesions of uterine cervix, as compared to the detection of HPV DNA by In Situ Hybridisation and by Hybrid Capture.

## MATERIAL AND METHODS

Paraffin blocks of 153 cervical biopsies (110 CIN1-3, 43 cervicitis) were retrieved from the files of Instituto Adolfo Lutz, Sao Paulo, corresponding to a series of patients with a previous report of epithelial lesions on a Papanicolaou smear. In all cases, colposcopy performed at Hospital Leonor Mendes de Barros from 1996-1997 was considered satisfactory, and biopsies were obtained from the abnormal areas of TZ. Biopsies were fixed in formalin, embedded in paraffin and routinely stained by hematoxylin-eosin. Histological diagnosis followed the criteria defined by WHO<sup>21</sup>. Non-isotopic In situ hybridisation (NISH) had been previously carried out, as reported by Roteli-Martins et al, 2001, using a digoxigenin-labelled wide spectrum (HPV 6/11, 16, 18, 31/33) cocktail probe with biotinylated tyramide-catalyzed signal amplification system (CSA- DAKO, USA). New 3 micrometer-sections from the paraffin blocks were coated on sylanised-slides and submitted to immunohistochemical (IHC) detection of an epitope of L1 protein common to High Risk HPV, by overnight (16 hs) incubation with mouse monoclonal antibody T16<sup>VAMP</sup>, from Virofem Diagnostica GmbH, Germany, previously reported as specific for HPV types 16, 18, 33, 35, 39, 45, 56 and 58<sup>19</sup>. Amplification of the reaction was performed with a streptavidin-biotin-peroxidase method (LSAB, DAKO, USA). Heat-induced epitope retrieval was performed in pressure cooker for 3 minutes, in a solution of citrate buffer pH6.0. Positive and negative controls were provided for NISH and for IHC. On colposcopy, vaginal and cervical swabs were taken for High-Oncogenic HPV-DNA detection (types 16, 18, 31, 33, 35, 45, 51, 52 and 56) by Hybrid Capture (Digene, Inc., USA). Samples were classified as positive if the relative light unit (RLU) at chemoluminometer was greater than the mean of the positive control values. Furthermore, an estimation of "viral load" was performed according to Cox et al, 1995: Samples with RLU>5 were considered as harboring a "high viral load" of HPV whereas those with 1<RLU<5 were considered as "low viral load". For statistical analysis, odds ratios (OR) with 95% confidence interval were computed to express the risk estimates<sup>1</sup>.

## RESULTS

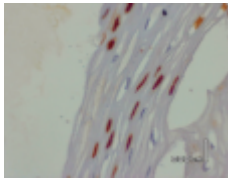
Out of 153 samples submitted to the High-Risk HPV L1 protein (HR-HPV L1), 35 were positive, 121 were negative. Positive reaction was characterized by a strong staining of the whole nucleus, surrounded by a cytoplasm with no background, except for rare cases with a granular cytoplasmic staining, possibly due to endogenous biotin or other endogenous component unmasked by the heat-induced epitope retrieval. In most cases, positive reaction with for HR-HPV L1 was positive in "classic" or "spindle" koilocytes or in dyskeratocytes, presenting nuclear characteristics for CIN 1 or for CIN 2, even in the samples of CIN 3 (fig 1B,1C,1D). The distribution of immuno-reactivity for HR-HPV L1 and HPV-DNA detection by NISH according to the histological diagnosis is depicted at Table 1, where it can be seen that some keratinocytes without histological HPV-associated lesion also immunoreacted HR-HPV L1 in 4 cases (9.3%) (fig 1A). Out of 110 CIN cases, HR-HPV L1 was immunodetected in 31 (28.2%). Non-isotopic In Situ Hybridization (NISH) yielded a nuclear staining of HPV-DNA in 54 cases. As depicted at Table 2, some keratinocytes without histological HPV-associated lesion also harboured HPV-DNA as detected by NISH in 6 cases (14.0%). Out of 110 CIN cases, HPV DNA was found in 48 samples (43.6%). A strong association was found between immunohistochemistry and Non-Isotopic In Situ Hybridization. The use of monoclonal antibody directed to HR-HPV L1 yielded an OR = 9.3 for a positive ISH reaction for HPV-DNA. Regarding the discrepancies between IHC for HR-HPV antibody and NISH, among 9 cases positive for IHC but negative for ISH, histopathological diagnosis was cervicitis in 1 case, CIN1 in 6 and CIN2 in 2 cases, whereas, out of 28 cases positive for NISH but negative for IHC, 3 were cervicitis, 11 CIN1, 8 CIN2 and 6 CIN3.

High-risk HPV DNA was also detected by Hybrid Capture in 72 out of 146 samples. Table 2 shows the relation between the immunohistochemical detection of HR-HPV L1 protein and HR-HPV-DNA by Hybrid Capture. The results from the two methods agreed in 65 negative and in 26 positive results. Thus, although less sensitive, IHC was also correlated to HC (OR=4.3). Regarding the estimation of "viral load" by Hybrid Capture, Two out of 16 cases with "low viral-load" HR-HPV by HC were positive for IHC, whereas, out of 56 "high viral-load cases", 24 immunoreacted HR-HPV L1 protein (OR= 3.6).

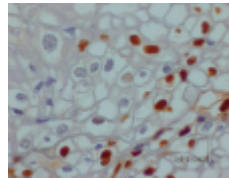
TABLE 1. Comparison of detection of HR-HPV L1 and HPV-DNA according to histological classification of epithelial lesions

Histological Diagnosis	HPV-DNA+ HR-HPV L1-	HPV-DNA+ HR-HPV L1+	HPV-DNA- HR-HPV L1+	HPV-DNA- HR-HPV L1-	TOTAL
Cervicitis	3	3	1	36	43
CIN1	11	10	6	37	64
CIN2	8	9	2	8	27
CIN3	6	4	0	9	19
TOTAL	28	26	9	90	153

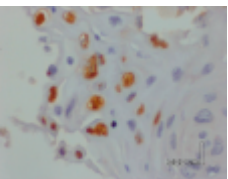
## Immunoreaction of HR-HPV L1 protein and cervical lesions



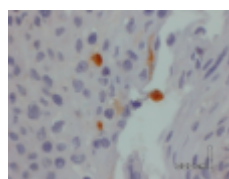
1A - Cervicitis



1B - CIN 1



1C - CIN 2



1D - CIN 3

## DISCUSSION

This study, designed to assess the immunoreactivity of L1 capsid protein of HR-HPV in a series of uterine cervix biopsies, showed nuclear immunoreaction of this antigen in 28.2% of patients with CIN, contrasting to 9.3% in samples with cervicitis.

Since it is well documented that the vast majority of cases of CIN harbor HPV DNA<sup>28</sup>, our findings show a low sensitivity of immunohistochemistry, which, thus, should not be used for screening for HPV detection. However, since morphological methods remain the gold-standard for the diagnosis of neoplasia and its precursors, the "in situ" detection of viral markers may offer useful information on biological meaning of histological patterns, as we have reported based on non-isotopic in situ hybridisation (NISH)<sup>19</sup>, and, especially, as hints for possible progression, as recently suggested by Melsheimer et al, 2002). Immunohistochemical detection of papillomavirus capsid protein was possible by the development of polyclonal antibodies by Jensen et al, 1980, leading to its identification in the nuclei of keratinocytes. Although several authors suggested the potential use of this approach in intra-epithelial neoplasia<sup>19</sup>, when it was demonstrated that only a sub-group of HPV types was more strongly associated to the development of carcinoma<sup>13</sup>, such immunohistochemical approach to HPV was less used since polyclonal antibodies did not yield to the identification of the High Risk-HPV. Since such a differentiation was achieved by the development of T16 monoclonal antibody by Sapp et al, 1994, several recent abstracts have been presented, showing, as we did herein, the immunoreaction of HR-HPV L1 protein in a subgroup of cases with CIN 1 and CIN 2.

The finding of 19 cases of CIN 1 or CIN2 positive for HPV-DNA by NISH, but not immunoreacting HR-HPV L1 protein could be ascribed either to a "latent phase" of infection or, more probably, to a lower sensitivity or the IHC amplification method used herein. Since NISH has become a more sensitive method when the catalyzing-signal amplification (CSA/CARD) was introduced<sup>19</sup>, further studies should assess the potential sensitivity of monoclonal antibody T16<sup>VAMP</sup> with this amplification system, preferentially in more recently collected tissue samples.

The rather low immunodetection rate of HR-HPV L1 protein in CIN 3 cases should possibly correspond to the integration of HPV DNA sequences in the genome of infected keratinocytes, thus truncating the expression of L1 gene<sup>11,12,23,25,27</sup>.

Immunodetection of HR-HPV-L1 in 4 cases of cervicitis in this series of women submitted to biopsy due to a recent finding of HPV-associated cytological alterations could possibly represent a moment of replicative HPV infection not yet followed by a cytopathic effect in this area, thus deserving future studies. Since in several cases studied here in HPV infection was only detected by one of the methods, NISH and IHC should preferentially be used combined.

A major potential clinical use of this immunodetection is its application in the initial sample of cohorts of cases initially diagnosed as CIN1, CIN2 or as with "equivocal lesions"<sup>17</sup>. If this approach proves our hypothesis that such immunoreaction might correspond to an active infection with a HR-HPV with a higher rate of evolution to higher-degree epithelial lesions, then its use as an adjunct method for selecting cases for a closer follow-up and earlier intervention should be considered.

## REFERENCES

- Agresti A. Categorical data analysis, p558. John Wiley Eds. New York.
- Alves V.A.F et al. Rev. Inst. Adolfo Lutz 1980; 40: 101-106.
- Alves V.A.F et al. J.Bras.Ginecol. 1988; 98: 257 - 261.
- Biner P et al. Mod Pathol. 2001 Jul; 14(7):702-9.
- Cox JT et al. Am J Obstet Gynecol 1995; 172:946-954.
- Griesner H et al. German Society for Pathology, Kiel, Germany, 2000, (7th-10th June).
- Gudat F et al. Lab. Invest. 1975; 32: 1-8.
- Habedank S et al. Virchows Archiv 436(3), 333, 2001
- Helsheimer P et al. Acta Cytologica, 2002 (accepted, in press)
- Jensen AB et al. J Natl Cancer Inst 1980; 64: 495-500
- Klaes R et al. Cancer Research 1999; 59: 6132-6136.
- Kuman RJ et al. Am. J. Obstet. Gynecol. 1981; 140: 931-935
- Lorincz AT et al. Obstet Gynecol 1992 Mar;79(3): 328-37.
- McMurray HR et al. Int J Exp Path 2001; 82: 15-33.
- Neumann H et al. 14th International Congress of Cytology, Amsterdam, Netherlands, 2001(27th-31st May)
- Payá CV et al. Hepatology. 1990 Jul;12(1):119-26.
- Richter RM, Nuovo G.J. ObstetGynecol. 1990 Feb;75(2):223-6.
- Roteli-Martins CM et al. Pathol. Res. Pract. 2001; 197: 677 - 682
- Sapp M et al. J. Gen.Virol. 1994; 75: 3375-3383
- Sapp M. HPV2001, 19th International Human Papillomavirus Conference, Florianopolis, Brasil, 2001(2nd-8th September)
- Scully RE et al. World Health Organization - International histological classification of tumors, 2nd ed, Springer-Verlag, Berlin, 1994
- Seedorf K et al. Virology. 1985 Aug;145(1):181-5.
- Stoler MH et al. Hum Pathol. 1992 Feb;23(2):171-28.
- Vargas PA et al. Histopathology. 2000 Aug;37(2):141-6.
- von Knebel Doeberitz M et al. Proc.Natl. Acad. Sci. USA 1991; 88: 1411-1415.
- Walboomers JM et al. J Pathol. 1999 Sep;186(1):12-9.
- Zur Hausen H. Nat Rev Cancer. 2002 May;2(5):342-50.