

ORIGINAL ARTICLE

Impact of HPV detection in colorectal adenocarcinoma: HPV protein and chromogenic in situ hybridization analysis based on tissue microarrays

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Summary

Purpose: Human papillomaviruses (HPV)-mediated cervical carcinogenesis represents a well analyzed model of viral implication in epithelial malignant transformation. Concerning colorectal cancer, HPV infection seems to be a significant genetic event in squamous colon epithelia carcinogenesis, but with an unclear role in colon adenocarcinomas (CACs). In the current study, we analyzed 60 CACs based on tissue microarray (TMA) blocks.

Methods: Cancerous tissues were cored, embedded on a tissue microarray block and analyzed by immunohistochemistry (HPV IHC) and also by chromogenic in situ hybridization (HPV 16/18 DNA CISH) in repetitive serial sections for protein and DNA specific typing detection, respectively.

Results: Based on HPV IHC and CISH simultaneous analysis, 16 (26.6%) cases expressed HPV protein. In 7 (11.6%) cores HPV 16/18 DNA signals were detected. Overall HPV protein expression and stage of the examined cases were significantly correlated with HPV CISH results ($p=0.0001$, $p=0.022$, respectively).

Conclusion: A subset of CACs demonstrated HPV infection associated with stage. In particular, detection of 16/18 HPV DNA types seemed to be a molecular parameter in analyzing genetically CACs, in contrast to HPV protein expression which did not offer significant and specific molecular information.

Key words: adenocarcinoma, colon, HPV, in situ hybridization, protein

Introduction

Viral mediated carcinogenesis - especially based on HPV- affects a variety of epithelia, including vaginocervical, vulvar, anal or oropharyngeal [1-3]. Extended molecular analyses have confirmed that high risk (HR) HPV type persistent infection - focused on uterine cervix squamocolumnar (SC) junction epithelia - leads to their cancerous transformation [4,5]. Precursor lesions include cervical intraepithelial neoplasia (CIN) or squamous intraepithelial lesions (SILs) based on pathological and cytological assessments, respectively [6-8]. Simple HPV infection is not the crucial

event during the carcinogenetic process [9,10]. HR HPV DNA integration into the host chromosome - regarding predominantly HPV 16/18/31/33/45/53 subtypes - modifies the human epithelial cell DNA by inactivating p53 and Rb gene pathways [11,12]. Development and improvement of HPV DNA tests based on polymerase chain reaction (PCR) or *in situ* hybridization (ISH) molecular techniques provide a significant approach in early detection of HPV infection, whereas m-RNA analysis is a useful tool for identifying the crucial overexpression of E6 and E7 HPV oncoproteins that stimulate the transcriptional phase inside the infected epithelial cells [13-15].

Although mechanisms of HPV-associated infection and carcinogenesis regarding genital epithelia seem to be well known, there is limited molecular knowledge concerning its implication and role in CAC genesis and progression [16,17]. In contrast to adenocarcinoma, anal-derived squamous cell carcinomas (SCCs) are characterized by increased HR HPV DNA levels due to sexual viral transmission [18,19]. Among HR HPV DNA subtypes detected in colorectal cancer cases, HPV 16 seems to be the major cause, followed by HPV 18 type [20,21]. In particular, in anal HPV-related SCCs there is a similar to uterine cervix CIN terminology, including multistep anal intraepithelial neoplasias (AINs), but there is no analogue regarding colon adenocarcinomas [22].

In the current study, we analyzed colon adenocarcinoma tissue microarray cores with a combination of IHC and CISH regarding HPV infection in the corresponding epithelia.

Methods

Sixty formalin fixed and paraffin embedded tissue samples of histologically confirmed CACs obtained by surgical resection (local or distant colectomies) between 2006 and 2009 were evaluated. The Department of Pathology (417 VA Hospital-NIMTS, Athens, Greece) and the local ethics committee gave permission to use these tissues for research purposes. Oral informed consent was obtained from each patient and the study protocol conformed to the ethical guidelines of the World Medical Association Declaration of Helsinki – Ethical Principles for Medical Research Involving Human Subjects as revised in Tokyo 2004. Ten microscopically normal-appearing colon epithelia samples were used as control group. All corresponding hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists for confirmation of diagnosis and classification according to the World Health Organization (WHO) grading criteria for CACs. Tumor staging was assessed using the Dukes staging classification.

Tissue microarray construction

Areas of interest were identified in H&E stained slides by conventional microscope (Olympus BX-50, Melville, NY, USA). Selection of these areas was made on the basis of tumor sufficiency, avoiding sites of necrosis or bleeding. Using ATA-100 apparatus (Chemicon International, Temecula, CA, USA), all of the source blocks were cored twice (in order to secure the presence of each case in the final blocks) and 1.5-mm diameter tissue cylindrical cores were transferred from each conventional donor block to the recipient blocks. After 3 mm microtome sectioning and H&E staining the final constructed TMA blocks contained 58/60 (96.6%) and 54/60 (90%) cores of tissue cylindrical specimens. We

observed microscopically that all examined cases were represented by at least one or two tissue spots (confirmation of the adequacy of the examined specimens).

Immunohistochemistry analysis

Ready-to-use mouse monoclonal anti- HPV L1 Cocktail Broad Spectrum (HPV-1, 6, 11, 16-16, 18 and 31/CAMVIR-1, BIOCARE, CA, USA) was applied on the corresponding TMA cores. IHC for HPV L1 antigen (dilution 1:100) was carried out on 3 µm serial sections of the corresponding tissue blocks. Two slides were deparaffinized and rehydrated. Both of them were heat-treated, followed by washing in distilled water. Following incubation with the primary antibody for 30 min, diaminobenzidine-tetrahydrochloride-DAB (0.03%) containing 0.1% hydrogen peroxide was applied as chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. IHC protocol was performed by the use of an automated staining system (I 6000 – Biogenex, San Ramon, CA, USA). Nuclear staining was observed regarding antigen expression. CIN tissue sections expressing HPV L1 protein were used as positive controls. For negative control slides, the primary antibody was omitted. Predefined cervical tissue sections expressing L1 protein and normal epithelia were used as control staining pattern.

Chromogenic in situ hybridization analysis

CISH analysis was based on ZytoFast kit (HPV 16/18 probe Digoxigenin-labeled / ZytoVision, GmbH, Br, Ge). Six µm serial sections of the corresponding TMA blocks were dewaxed, proteolyzed and then pipetted with 10 µl vortexed probe. After this procedure, the slides were covered by a coverslip. Then, they were denatured at 75°C for 5 min, transferred to a humidity chamber and hybridized for 60 min at 37°C. Positive reactivity for HPV type 16/18 DNA in epithelial cells was indicated by a distinctly stained blue nucleus. CIN tissue sections with HPV DNA signals were used as positive controls. Visualization of signals was finally performed by a conventional bright-field microscope using a 10x to 20x objective. For signal evaluation, necrotic, degenerated or overdigested cells were avoided as these cells often stain nonspecifically.

Statistics

Continuous data were presented as mean ± standard deviation, whereas categorical data as absolute and relative frequency. Several variables were examined including age, gender, tumor size, stage, and grade. Mann-Witney U test and also Student's t-test for continuous data in two independent samples were performed. Significance level was set at p=0.05. The STATISTICA statistical package (version 10.0, Stasoft Inc, USA) was used to analyze the data.

Results

Based on HPV IHC and CISH simultaneous analysis on the TMA cores, 16/60 (26.6%) cases expressed strongly HPV L1 protein (Figures 1 and 2). In 7/60 (11.6%) tissue cores HPV 16/18 DNA signals were detected as blue signals covering the corresponding nuclei areas (Figures 2 and 3). Overall HPV protein expression correlated with stage (mainly Dukes A-B stage) of the examined cases ($p=0.022$). Significant statistical association was also identified associating HPV CISH to HPV IHC overall results ($p=0.0001$). In addition, stage vs tumor size was found to be marginally significant ($p=0.051$). No other significant values were observed correlating IHC/CISH results with grade, anatomic location/tumor size, gender and also age. Clinicopathological data and combined HPV IHC& HPV DNA CISH results are described on Table 1.

Discussion

HPV infection and especially its DNA integration into the corresponding epithelial infected nuclei is a significant genetic event that induces carcinogenesis in cervical epithelia [23]. Concerning CAC, some studies demonstrate negative results regarding HPV influence in host-colon cells, whereas others support an unclear, but significant role in colon carcinogenesis [24-26]. Despite these controversial results, identification of HPV DNA in colon epithelia adds molecular information regarding the genetic profile of the disease in subgroups of patients [27].

Detection of HPV DNA in infected epithelia is generally based on ISH or PCR techniques [28,29]. Immunohistochemical protocols have also been developed for detecting HPV L1 capsid protein, but the overall sensitivity of this method is lower comparing to strict molecular analyses [30,31]. Interestingly, chromogenic ISH analysis uses an immunohistochemical type reaction for HPV DNA signal-detection [32]. This technique is characterized by significant advantages regarding interpretation of viral status into host-epithelia cells, including conventional bright field low magnification (40 \times) DNA detection and also simultaneous tissue/cell morphology with genetic events [33,34]. Although extended HPV DNA typing is based predominantly on PCR protocols, ISH method provides fast accurate results that are friendly to conventional bright-field cytology examination [35].

In the current study we identified cases of CAC that demonstrated HPV infection, especially HR-types, such as 16/18. Comparing these results to IHC HPV L1 analysis we concluded that molecular detection provides a higher sensitivity although the overall HPV IHC and ISH correlation was statistically significant. In addi-

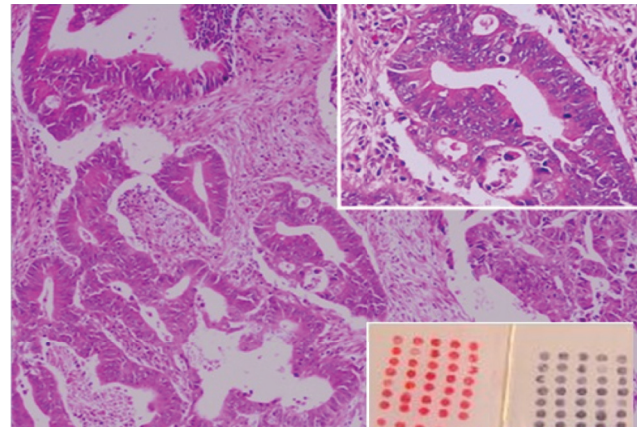


Figure 1. Colon adenocarcinoma tissue cores. H&E and HPV L1 stains (original magnification 10x, inside right up photo 40x).

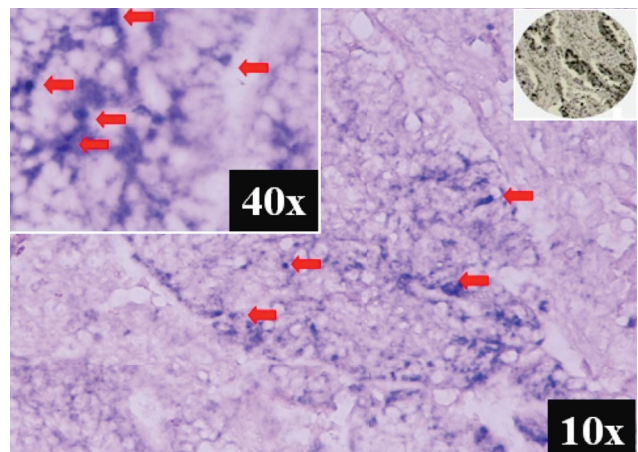


Figure 2. HPV DNA CISH analysis in colon adenocarcinoma. Blue areas (red arrows) represent virus DNA copies (original magnification 10x, 40x). HPV L1 expression in a tissue core (inlet right up photo: nuclear staining, original magnification 10x).

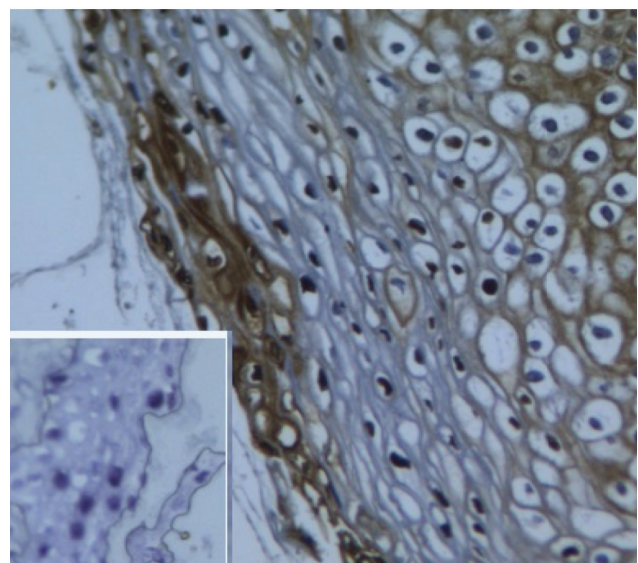


Figure 3. HPV L1 expression combined with DNA *in situ* hybridization analysis (inlet) in a case of CIN I used a positive marker for HPV IHC & CISH (original magnification 40x).

Table 1. Clinicopathological data and combined HPV IHC & HPV DNA CISH results

(N=60)	HPV L1 IHC			HPV 16/18 CISH		
	Negative (N=44) %	Positive (N=16) %	p-value	Negative (N=53) %	Positive (N=7) %	p-value
Gender			NS			NS
Female (N=32)	66.7	34.3		28.1	71.9	
Male (N=28)	82.2	17.8		21.4	78.6	
Tumor diameter (cm)			NS			NS
0-3 (N=15)	87.3	12.7		91.7	8.3	
4-6 (N=30)	89.1	10.9		79.9	21.1	
≥7 (N=15)	92.2	7.8		88.4	11.6	
Grade			NS			NS
I (N=22)	88.4	11.6		93.4	6.6	
II (N=31)	10	90		95	5	
III (N=7)	98.4	1.6		100	0	
Dukes stage			NS			0.022
A (N=8)	93.4	6.6		95	5	
B (N=26)	90	10		95	5	
C (N=26)	90	10		98.4	1.6	
Location						
Other than rectum-sigmoid (N=24)	88.4	11.6		95	5	
Rectum-sigmoid (N=36)	85	15		93.4	6.66	
Overall HPV IHC vs HPV DNA*	0.001					

NS: not significant, p-value derived from Student's t-test and Mann-Whitney-Wilcoxon test* for independent samples

tion, the stage of the examined tumors was associated to HPV infection. Similar studies based on a combination of two molecular techniques (PCR – ISH or PCR – Southern blot hybridization) have provided evidence of HPV DNA implication in transforming the normal glandular cells of the colorectal mucosa to neoplastic, especially under the influence of HPV 16/18 or 33 HR-types [36,37]. Furthermore, quite recent molecular analyses have shown potential HPV16 E6 oncoprotein involvement in p53 inactivation in colorectal cancer and also that the transmission of HPV to the colon and rectum might occur through peripheral blood lymphocytes [38]. Experimentally, also, a microtubule regulatory protein, called stathmin, seems to play a critical role in survival of both normal and cancer cell lines lacking the tumor suppressor p53. Comparing specific colon cancer cell lines differing in TP53 genotype, a study group observed that stathmin depletion resulted in significant death only in cells lacking p53 [39].

Interestingly, also, another experimental study based on a combination of K-ras mutations analysis and HPV DNA typing PCR amplification and hybridization concluded that infection with HR HPV types and mutational activation of the K-ras gene are frequent and overlapped in subgroups of patients' genetic events in colorectal carcinogenesis [40]. So, overactivation of the ras gene family in conjunction with p53 inactivation - due to HPV DNA host-cell chromosome integration - seem to be closely related gene abnormalities, not only in cervical cancer but also in squamous cell carcinoma of the colon and similarly in colon or even lung adenocarcinoma [41].

In conclusion, our study showed that a subgroup of patients with CAC demonstrated HPV infection (concerning HR-types, predominantly HPV 16/18). Furthermore, ISH analysis was characterized by a superior sensitivity in detecting HR-HPV types comparing to protein (HPV L1) expression.

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