

Association of Human Papillomavirus and Colon Neoplasms

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• Human papillomavirus has been shown to be associated with squamous carcinomas. We evaluated benign and malignant colon tissues for the presence of human papillomavirus infection to determine if a similar relationship exists between human papillomavirus and colon neoplasms. Colon tissues were screened using an immunohistochemical technique to detect human papillomavirus antigen. In situ DNA hybridization was then performed on those tissues that yielded positive results by immunohistochemistry. Groups were compared using χ^2 analysis. Human papillomavirus antigen was present in 23% of normal colon specimens, 60% of benign tumors, and 97% of carcinomas. Human papilloma viral genome was demonstrated in 27% of benign tumors and in nearly 43% of all carcinomas tested. These data indicate that human papillomavirus infects the columnar mucosa of the colon, and that an association exists between human papillomavirus and colon neoplasia.

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The incidence of adenocarcinoma of the colon and rectum has remained unchanged for the last 40 years. Despite perioperative improvements in nutrition, antibiotics, and anesthesia, the mortality associated with colon carcinoma continues to be significant; approximately half of the 150 000 newly diagnosed patients with colorectal cancer this year will die of their disease. The causes of this neoplasm remain unknown. Despite associations of viral infections with other neoplasms for over three quarters of a century, a viral causative agent for colon cancer has not been proposed. Squamous cell carcinomas of the urogenital region and aerodigestive tract have previously been associated with human papillomavirus (HPV) infection and these relationships have proven clinically useful in both the diagnosis and monitoring of these diseases. Demonstration of a similar relationship in colon cancer may have the same effect on this disease.

We have recently demonstrated an association between HPV antigen and both benign and malignant neoplasms of the colon.^{1,2} These studies, however, only demonstrated virus-related proteins and not the viral genome. In situ DNA hybridization is a quick and reproducible test for those viral genomes and thus is specific for viral infections.

We chose to screen colon tissues, including normal mucosa, benign tumors, and cancers for HPV infection using an immunohistochemical technique to detect HPV antigen. In situ DNA hybridization was then utilized to demonstrate HPV genome in those colonic tissues that tested positive for viral antigen.

PATIENTS AND METHODS

Tissue for study was obtained from the Anatomical Pathology Section of the Veterans Affairs Medical Center, Reno, Nev. Thirty normal colons, 30 single tubulovillous adenomas, and 43 carcinomas (30 invasive carcinomas and 13 carcinomas in situ) were selected. Archival paraffin-embedded biopsy specimens of these tissues were studied. Immunohistochemical techniques were used to screen the tissue for the presence of HPV antigen. Specimens testing positive for the antigen underwent DNA hybridization. Four-micrometer sections of the tissue were cut and mounted on slides prepared with poly-L-lysine. Tissues were then deparaffinized in xylene and rehydrated in a series of alcohol washes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol. After rinsing in distilled water, the specimens were covered with 5% normal goat serum in phosphate-buffered saline (PBS) and incubated for 20 minutes. All incubation was carried out at room temperature in a closed humid chamber. The goat serum was decanted and rabbit anti-bovine papillomavirus antibody (1:100 μ L dilution in 1% albumin in PBS) was applied and incubated for 18 to 20 hours. The specimens were next washed in PBS, and a biotinylated horse anti-rabbit IgG (1:100 μ L dilution) was applied and incubated for 60 minutes. Tissue was again washed in PBS; then avidin-biotin complex (Vector Inc, Burlingame, Calif) was applied to the sections and incubated for 60 minutes. Following washes in PBS, tissues were stained with 3,3'-diaminobenzidine tetrahydrochloride for 10 minutes and counterstained in hematoxylin for 1 to 3 minutes. Tissues were dehydrated with increasing concentrations of ethanol, cleared with xylene, and mounted. Resultant slides were interpreted blindly by a pathologist and graded simply as positive or negative.

Specimens that reacted positive by immunohistochemistry were subjected to in situ DNA hybridization. Four-micrometer sections of tissue were deparaffinized with xylene and rehydrated using a series of alcohol washes. Tissues were then subjected to protease digestion using a commercially prepared digestive reagent (Life Technologies, Gaithersburg, Md) for 20 minutes at 37°C, and then washed with buffered saline solutions prior to DNA hybridization. Specimens were reacted with pooled DNA probes to HPV virotypes 6, 11, 16, 18, 31, 33, and 35 (Life Tech) at 100°C for 5 minutes, then hybridization was allowed to occur at 37°C for 2 hours. Specimens were again washed in buffered saline solutions (Life Tech) for 3 minutes and counterstained. Tissues were then dehydrated in a series of increasing concentrations of alcohol, washed in xylene, and mounted on slides for photomicrography.

RESULTS

Human papillomavirus antigen was demonstrated in 97% of the carcinomas (Fig 1). The antigen was seen in 60% of the benign tumors (Fig 2) and 23% of the normal specimens (Fig 3). Despite small numbers ($n = 103$), the null hypothesis that these subpopulations were homogenous in their incidence of viral antigen was able to be rejected at $P < .005$ by χ^2 analysis. The viral genome was demonstrated in roughly one third of the carcinoma specimens tested (Fig 4), and two thirds of carcinomas in situ reacted positive for the virus. A smaller

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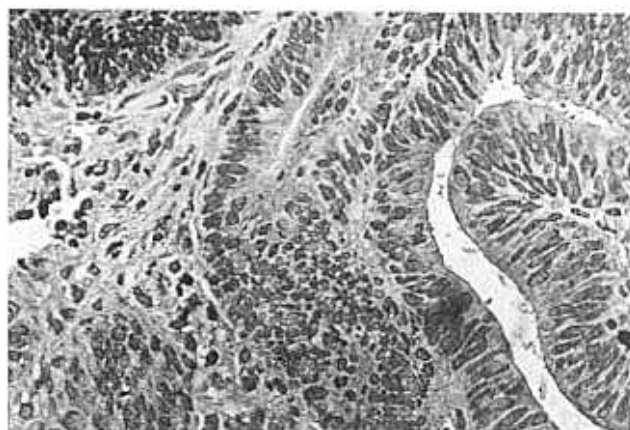
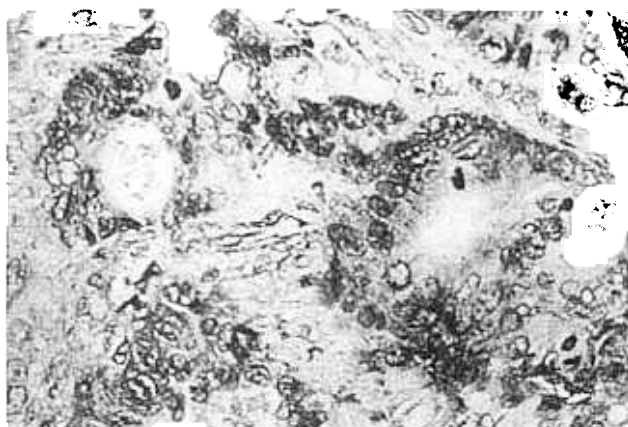


Fig 1.—Left, Photomicrograph of human colon carcinoma demonstrating human papillomavirus antigen by an immunohistochemical technique. The diaminobenzidine tetrahydrochloride stains brown in the presence of antigen-antibody complex (hematoxylin, original magnification $\times 100$). Right, Photomicrograph of human colon carcinoma that tested negative for human papillomavirus antigen. Reaction in the interstitium is due to nonspecific peroxidase within plasma cells (original magnification $\times 100$).

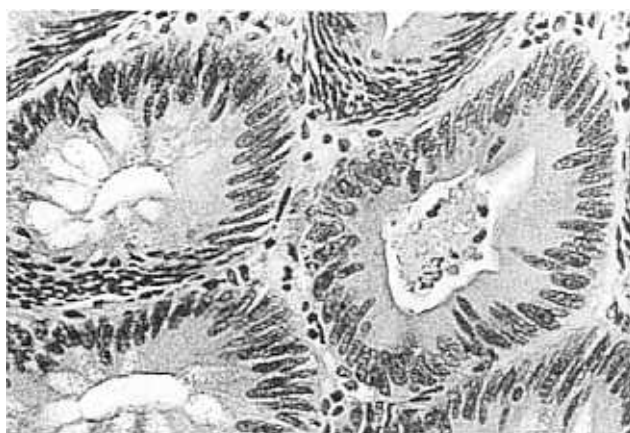
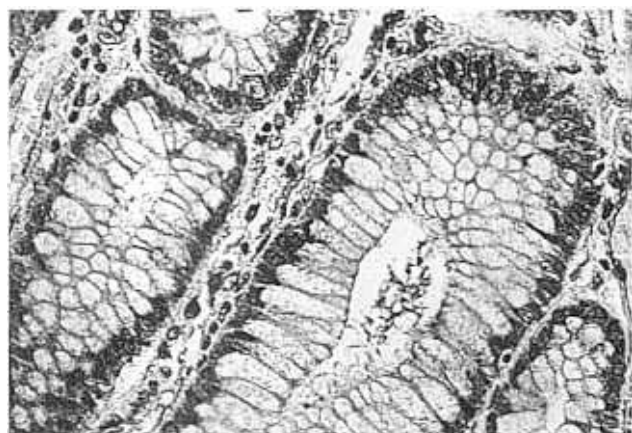


Fig 2.—Left, Photomicrograph demonstrating human papillomavirus antigen in a benign tubulovillous adenoma (original magnification $\times 100$). Right, Photomicrograph of a human tubulovillous adenoma testing negative for human papillomavirus antigen (original magnification $\times 100$).

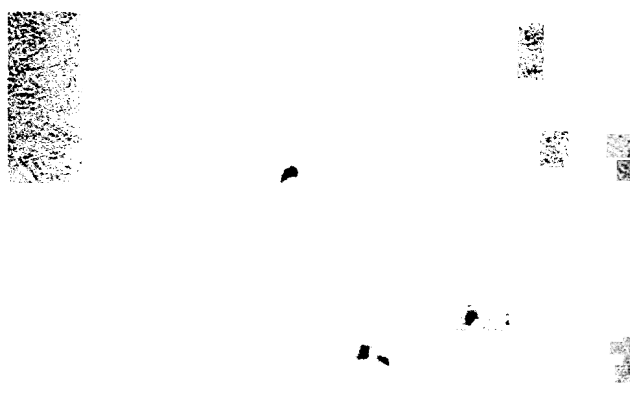
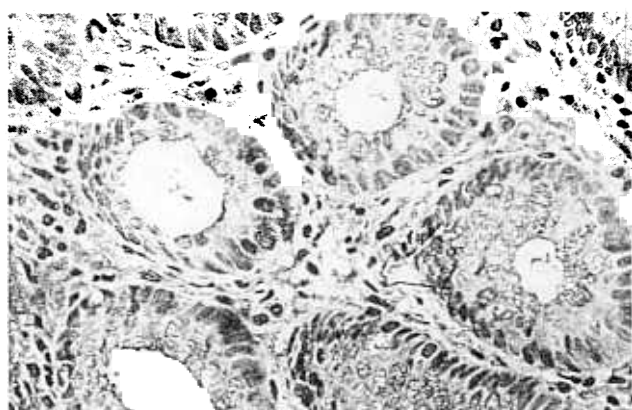


Fig 3.—Photomicrograph of normal human colon mucosa demonstrating human papillomavirus antigen (original magnification $\times 100$).

fraction of the benign tumors and none of the normal mucosa samples tested positive for the viral genome (Table).

COMMENT

The carcinogenic potential of the papillomavirus has been previously demonstrated,⁸ and progression from a benign laryngeal papilloma to carcinoma has been described.⁴ The mechanism by which oncogenesis occurs is not known, but retroviruses and DNA viruses are known to activate proto-oncogenes.⁵ This is probably a multistep process with dietary^{6,7} and genetic interactions.^{8,9} Evidence is accumulating that supports a causal role for HPV in benign and malignant lesions of the urogenital tract and the head and neck region. Carcinoma of the cervix,¹⁰⁻¹³ aerodigestive tract,¹⁴⁻¹⁷ lung,¹⁸ and esophagus^{19,20} have all been shown to have an association with HPV. Identification of this viral association has allowed for changes in diagnostic and therapeutic approaches to these diseases.

Proto-oncogene abnormalities have also been identified in colon neoplasia, including the *c-RAS* and *c-MYC* oncogenes.²¹ Products of the *c-MYC* oncogene have been demonstrated by immunoperoxidase-staining techniques in benign colonic polyps and carcinomas of the colon. Hybridization techniques have demonstrated the *c-MYC* oncogene in as many as 20% of colon cancers.²² Human papillomavirus is believed to incorporate into the host genome near the *c-MYC* oncogene, at least in carcinomas of the uterine cervix.²³ Evidence that oncogenes are active in colon neoplasia adds strength to the argument of multistage carcinogenesis and is consistent with the well-acknowledged progression from mucosa to adenomatous change to malignant degeneration (the polyp-cancer sequence).

We have previously demonstrated HPV antigen to be associated with colonic neoplasia. This association led us to investigate whether the virus was actually present in colon tumors or whether some other tumor antigen might have cross-reacted with the polyclonal antibody used in the immunohistochemical procedure. Hybridization of DNA remains the standard technique for the demonstration and thus identification of viral infections, but conventional extraction DNA hybridization is cumbersome and time consuming. In situ DNA hybridization has recently been described for detection of viral genome,²⁴ and was chosen for this study based on its previous success in similar situations. It has been shown to be a clinically useful technique in detecting HPV genome in

Results of Immunohistochemical Analysis and In Situ Hybridization*		
Tissue Type	No. (%) Positive for HPV Antigen	No. (%) Positive for HPV Genome
Normal mucosa (n = 30)	7/30 (23)	0/7
Tubulovillous adenomas (n = 30)	18/30 (60)†	5/18 (27)
Invasive carcinomas (n = 30)	29/30 (97)†	9/29 (31)
Carcinomas in situ (n = 13)	13/13 (100)†	9/13 (69)

*HPV indicates human papillomavirus.

† $P < .001$ by χ^2 compared with normal mucosa.

laryngeal papilloma tissue²⁵ and cytomegalovirus in colon tissue of immunosuppressed individuals.²⁶

The immunohistochemical demonstration of viral capsid protein in one of four normal mucosal specimens suggests a widespread prevalence of the virus. Extrapolated to the estimated US population of 250 million, a 23% prevalence would imply that nearly 60 million American colons harbor the virus. Our tissue samples were from a geriatric population, and we have no data on how or when viral infestation occurs, but such a large number is consistent with the emerging knowledge of this virus.

The detection of HPV genome in nearly half of the cancers tested clearly demonstrates the presence of the virus in these carcinomas. This validates the immunohistochemical data that detected viral capsid antigen in these lesions, and corroborates previous reports that identified the viral antigen in colon polyps. It is not clear why all of those tissues that displayed the viral capsid protein did not also demonstrate the viral genome; either the HPV associated with those tumors is one of the other 53 known virotypes not represented in our pooled probes or the virus is present in such low concentration that it is undetectable by in situ hybridization.

We have demonstrated the presence of HPV in the columnar mucosa of the colon. Further, we have described an increasing association of the virus with progressive degrees of malignant degeneration of that mucosa. The potential therapeutic and diagnostic implications of this association remain to be established, but we believe that this association with HPV will have an impact on future understanding of both the development of colon carcinoma and its diagnostic and therapeutic interventions.

References

- Kirgan D, Manalo P, McGregor B. Immunohistochemical demonstration of human papillomavirus antigen in human colon neoplasms. *J Surg Res*. 1990;48:397-402.
- Kirgan D, Manalo P, McGregor B. Distribution of human papillomavirus in colon neoplasia. Presented at the annual meeting of the International Society of Surgery, September 12, 1989, Toronto, Canada.
- Rous P, Kidd JG. The carcinogenic effect of a papillomavirus on the tarred skin of rabbits. *J Exp Med*. 1938;67:399-428.
- Zarod AP, Rutherford JD, Corbitt G. Malignant progression of laryngeal papilloma associated with human papillomavirus type 6 (HPV-6) DNA. *J Clin Pathol*. 1988;41:280-283.
- Land H, Parada L, Weinberg R. Cellular oncogenes and multistep carcinogenesis. *Science*. 1983;18:771-777.
- Reddy BA, Wynder EL. Large-bowel carcinogenesis: fecal constituents of populations with diverse incidence rates of colon cancer. *J Natl Cancer Inst*. 1973;50:1437-1442.
- Burkitt DP. Etiology and prevention of colorectal cancer. *Hosp Pract Off*. 1984;19:67-77.
- Cannon-Albright LA, Skolnick MH, Bishop DT, Lee RG, Burt RW. Common inheritance of susceptibility to colonic adenomatous polyps and associated colorectal cancers. *N Engl J Med*. 1988;319:533-537.
- Kune GA, Kune S, Watson LF. The role of heredity in the etiology of large bowel cancer: data from the Melbourne colorectal cancer study. *World J Surg*. 1989;13:124-131.
- Kurman RJ, Shah KH, Lancaster WD, Jenson AB. Immunoperoxidase localization of papillomavirus antigens in cervical dysplasia and vulvar condylomas. *Am J Obstet Gynecol*. 1981;140:931-935.
- Lancaster WD, Jenson AB. Evidence for papillomavirus genus-specific antigens and DNA in laryngeal papilloma. *Intervirology*. 1981;15:204-212.
- Durst M, Gissmann L, Ikenberg H, zur Hausen H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proc Natl Acad Sci USA*. 1983;80:3812-3815.
- Crum CP, Ikenberg H, Richart RM, Gissman L. Human papillomavirus type 16 and early cervical neoplasia. *N Engl J Med*. 1985;310:880-883.
- Kashima H, Wu T-C, Mounts P, Heffner D, Cachay A, Hyams V. Carcinoma ex-papilloma: histologic and virologic studies in whole-organ sections of the larynx. *Laryngoscope*. 1988;98:619-624.
- Corbitt G, Zarod AP, Arrand JR, Longson M, Farrington WT. Human papillomavirus (HPV) genotypes associated with laryngeal papilloma. *J Clin Pathol*. 1988;41:284-288.
- Brandsma JL, Steinberg BM, Abramson AL, Winkler B. Presence of human papillomavirus type 16 related sequences in verrucous carcinoma of the larynx. *Cancer Res*. 1986;46:2185-2188.
- Gissmann L, Dielz V, Schultze-Coulon HJ, zur Hausen H. Molecular cloning and characterization of human papillomavirus DNA derived from a laryngeal papilloma. *J Virol*. 1982;44:393-400.
- Syrganen K, Syrganen S, Kellokoski J, Karja J, Mantyjarvi R. Human papillomavirus (HPV) type 6 and 16 DNA sequences in bronchial squamous cell carcinoma demonstrated by in situ DNA hybridization. *Lung*. 1989;167:33-42.

19. Winkler B, Capo V, Reumann W, et al. Human papillomavirus infection of the esophagus. *Cancer*. 1985;55:149-155.
20. Kulski J, Demeter T, Sterrett G, Shilkin K. Human papillomavirus DNA in oesophageal carcinoma. *Lancet*. 1986;2:683.
21. Stewart J, Evan G, Watson J, Sikora K. Detection of the c-MYC oncogene product in colonic polyps and carcinomas. *Br J Cancer*. 1986;53:1-6.
22. Meltzer SJ, Ahnen DJ, Battifora H, Yokota J, Cline M. Protooncogene abnormalities in colon cancers and adenomatous polyps. *Gastroenterology*. 1987;92:1174-1180.
23. Broker T, Thomas R. Structure and genetic expression of papillomavir-

uses. *Obstet Gynecol Clin North Am*. 1987;14:329-348.

24. Brigati D, Myerson D, Leary J, et al. Detection of viral genomes in cultured cells and paraffin-embedded tissue sections using biotin-labeled hybridization probes. *Virology*. 1983;126:32-50.

25. Terry R, Lewis F, Griffiths S, Wells M, Bird C. Demonstration of human papillomavirus types 6 and 11 in juvenile laryngeal papillomatosis by in situ DNA hybridization. *J Pathol*. 1987;153:245-248.

26. Roberts W, Hammond S, Sneddon J, Thesing J, Caldwell J, Clausen K. In situ DNA hybridization for cytomegalovirus in colonoscopic biopsies. *Arch Pathol Lab Med*. 1988;112:1106-1109.

Discussion

MARVIN M. ROMSDAHL, MD, PhD, Houston, Tex: Dr Kirgan and his associates have examined benign and malignant colonic tissue for HPV and have provided evidence that probably should be interpreted as preliminary in regard to an association between HPV and colon cancer. Clearly the issue of colon cancer causation by HPV is neither made nor implied.

Human papillomavirus has previously been rather strictly associated with squamous epithelium, either in the oral pharynx or the anorectal regions. Demonstration of HPV in glandular epithelium would be a new observation. If indeed this should prove to be the case, and particularly if associated with glandular-type carcinoma, a major scientific finding would be at hand. A confirmed association to colon cancer, even without reference to causation, would be extremely important.

This type of study will receive extreme scrutiny in regard to technology employed and its interpretations because of the inconsistency of these findings with generally accepted concepts of colon carcinogenesis.

The authors have previously shown virus-associated proteins in colonic tissues, including benign and malignant neoplasms, by histochemical techniques. They then proceeded to employ DNA hybridization to detect whether the virus was contained in the nucleus of the different colon tissues. While the immunohistochemical evidence is reasonably evident, a more exact and specific test for HPV concerns the DNA hybridization, which they indeed have done. Now, in regard to DNA hybridization, I would like to point out that there are several techniques to show this particular phenomenon.

The first technique and the oldest is the in situ labeling, using tissue sections, such as they have done. A second technique that has been developed is termed the "dot blot" technique. For this test, homogenates of the tissue that you wish to examine, such as rectal mucosa, colon cancer, or polyps are allowed to react with your indicator DNA, which would, of course, come from any of the virus subtypes. This, then, can be tested either immunochemically or by radioimmunoassay.

The final type of test for DNA hybridization concerns what is known as the "southern blot" technique. This is a test in which a homogenate of the test tissue is submitted to gel electrophoresis and then radioimmunoassay is used to detect whether indeed there has been hybridization between the papillomavirus and the DNA contained in the colonic tissues. Of these different techniques, the southern blot technique is the ultimate standard and should be employed by the authors to document their findings at this time.

The authors did test seven different HPV viral types, but under low stringency. It would be worthwhile to test these same viral types under high stringency. Perhaps in their closing remarks they will

discuss stringency for this audience and possible advantages that high stringency might have in adding credibility to their scientific observations.

The observations that Dr Kirgan and his associates have made may have significant implications in colon cancer, should an undisputed association with HPV be forthcoming. However, this is a revolutionary concept and I believe that use of the highest scientific technology has to be employed to support their findings.

Finally, it is a pleasure to see general surgeons and surgical trainees familiarizing themselves with and engaging in basic research of conditions we regularly encounter, such as colon cancer.

DAVID L. BOUWMAN, MD, Detroit, Mich: Do you have any cases in which, in a single individual, assays were run on normal colonic mucosa as well as the villous adenoma, tubular adenoma, or carcinoma?

GEORGE B. MARKLE IV, MD, Carlsbad, NM: I have long been impressed with the similarities between colon cancer and carcinoma of the cervix: dysplasia first, then going into carcinoma in situ, and finally frank carcinoma. So there's a similarity here. Where does this virus come from if we are dealing with a papillomavirus? In gynecology we know where it comes from and we can do something about it. Are there any ideas as to how this virus gets into the gut?

DR MCGREGOR: We have many of the same concerns expressed by Dr Romsdahl and other reviewers. To be sure, gene amplification and southern blot techniques are the state of the art, even though those filtration techniques would not have allowed the simultaneous identification and localization as did the in situ hybridization.

Dr Bouwman, we had no cases in which all three were involved. Second, all of the "normal" mucosa described was taken from patients with nonmalignant diseases. Dr Markle, we have speculated about the origin of this virus and have completed a distribution study of tubulovillous adenomas with the theory that if there were a gradient from rectum to cecum, a retrograde transmission from the perineum would be suggested, but if the distribution were homogenous, then a viremic presentation would be more likely. The result of that study was that viral distribution was uniform throughout the colon.

But at the basis of all the skepticism, especially among surgeons is this: Do we believe all this fancy biochemistry? The validation of any such observation is that it allows us to predict future observations. One of those predictions surely would be that if it is there, we ought to be able to see it. Indeed, we have recently been able to demonstrate: (1) central clearing of chromatin, (2) openings in the nuclear envelope characteristic of a lytic virus, and (3) 45 to 55 nm viral particles (the size of HPV) all within the nucleus of a cell of a human colon adenocarcinoma. We believe that this is a real phenomenon and is worthy of further study.