Characterization of Integrated Human Papillomavirus Type 11 DNA in Primary and Metastatic Tumors from a Renal Transplant Recipient

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ABSTRACT

A primary perianal squamous cell carcinoma and two metastatic tumors from a renal transplant recipient with a previous history of condyloma acuminatum were analyzed by filter hybridization for the presence of human papillomavirus (HPV) DNA. Each of the DNA extracts from these three tissues was found to contain HPV DNA. Stringent hybridization and restriction endonuclease analysis identified this viral DNA as HPV 11 related, which largely comigrated with cellular DNA, suggesting the presence of integrated viral DNA. Each DNA extract was analyzed by two-dimensional gel electrophoresis, which separates circular and linear forms of DNA and can demonstrate linear viral DNA, which comigrated with high molecular weight linear cellular DNA, thus implying viral integration. In all three cases the vast majority of viral DNA was found to comigrate with linear DNA; in addition, a significant portion comigrated with high molecular weight cellular DNA, suggesting the presence of integrated viral DNA in these tumors. Restriction endonuclease analysis of high molecular weight cellular DNA from each of these tumors revealed identical banding patterns, indicating that the integration site in each tissue is identical and, therefore, that all three tumors most likely originated from a single clonal event. These molecular results are presented in light of the clinical history of this patient with a histologically "low grade," but biologically aggressive, squamous cell carcinoma and suggest that HPV 11 may be associated with the initiation of malignant epithelial neoplasms.

INTRODUCTION

It has been well documented that infection with certain types of HPV is often associated with malignant epithelial tumors of the anogenital region. The predominant types associated with these malignant lesions include HPV 16, 18, and 31 (1-3), and to a lesser degree HPV 33 and 35 (4, 5), often found integrated into the host genome. In contrast, HPV 6 and HPV 11 DNA has been observed to exist as free episomal genomes in benign genital tumors and in laryngeal papillomas (6, 7). HPV 11 has also been detected apparently as an episomal component in a penile squamous cell carcinoma (8) and in nonmetastasizing penile verrucous carcinoma (9). In one case of a particularly aggressive verrucous carcinoma, an HPV 6 variant has been detected (10). In a series of patients treated with radiation therapy for laryngeal papillomas, malignant transformation of the virally infected epithelium has been observed (11, 12). In general, however, in the absence of "promoting factors" such as radiation, cells infected with HPV 6 and HPV 11 have been widely regarded as incapable of producing malignant tumors de novo.

We have studied the role that specific papillomaviruses play in the pathogenesis of benign and malignant epithelial tumors of the anogenital region in normal (immunocompetent) and immunosuppressed patients, such as renal transplant recipients (9, 13-17). Anogenital tumors occur with greater than a 100-fold incidence in renal transplant recipients, as compared with the general population, and generally behave in a biologically aggressive manner (18). However, malignant tumors of the anogenital region in these patients rarely metastasize widely, but rather invade locally, with a high propensity to recur after excision (19). This report analyzes the unique clinical, pathological, and molecular findings of an aggressive perianal squamous cell carcinoma containing a HPV 11 genome that is integrated into the host cellular DNA in the primary and metastatic tumors occurring in a single renal transplant patient.

PATIENT, MATERIALS, AND METHODS

Case Report

The patient was a 44-year-old native American male who developed chronic renal failure in 1978, subsequent to an episode of postinfectious glomerulonephritis. At the time of his medical evaluation, his medical history revealed an incidence of diabetes mellitus and tuberculosis in other family members; on physical examination, he was notably hypertensive and oliguric. Renal transplantation with a double HLA antigen allograft was performed, and the patient recovered without complication. Several weeks later, however, he developed vascular and tubulointerstitial rejection. Treatment for rejection with antithymocyte globulin and prednisone was successful, and the patient maintained adequate renal function until 1983, at which time he again presented with signs of pyelonephritis and allograft rejection. Noted during hospitalization were several perianal "warts," which were not treated at that time. Renal function failed to improve with treatment and, consequently, immunosuppression was discontinued, necessitating the patient to reinstitute hemodialysis.

A second renal transplant was performed in 1986, which was complicated by the development of a draining incisional hernia. During localized allograft radiation treatment, no exposure was given to the anal area. Following discharge from the hospital, the patient underwent surgical excision of the mass of perianal warts, which microscopically was consistent with condyloma acuminatum. In late 1986, the patient was hospitalized with fever, hypotension, and massive abdominal ascites. Examination of the perianal mass demonstrated a 6.0-cm cauliflower tumor, which was partially necrotic. A biopsy at this time was reported to be a well differentiated squamous cell carcinoma. Computer-assisted tomography of the chest and abdomen revealed two pulmonary nodules and a large lesion in the right lobe of the liver. Additionally, laboratory studies showed a serum calcium of 14.5 mg/dl, normal parathyroid hormone levels, and multiple organisms growing in blood culture. The patient died a short time later with septic shock and bacterial peritonitis.

The autopsy examination revealed 1.8 liters of serosanguinous ascites and dense fibrinous adhesions enveloping the abdominal organs. The perianal tumor was noted to infiltrate into the subcutaneous tissue and was extensively necrotic. Within the abdomen, several pelvic lymph nodes contained tumor, and the liver was subtotally replaced by gray and firm tumor nodules of various sizes. Two circumscribed tumor masses in the left lower lobe of the lung had the same gross characteristics. Histopathologically, tumor from each body site was identical and was composed of complex and irregular nests of cells surrounding keratin pearls, admixed in a loose fibrous stroma (Fig. 1A). Areas of necrosis were present throughout the tumor nodules. Individual tumor...
cells were variably large and small, with copious eosinophilic cytoplasm and pleomorphic nuclei, often with prominent perinuclear clearing (Fig. 1, B and C). Chromatin was vesiculated and nucleoli were indistinct. Immunohistochemical stains were strongly positive for cytoplasmic and extracellular cytokeratin (Fig. 1D) but were uniformly negative for parathyroid hormone and papillomavirus antigens, using a commercially available detection kit (Dakor, Inc.). These findings were interpreted as indicating a well differentiated squamous cell carcinoma arising in the perianal tissue and metastasizing distantly to the pelvic lymph nodes, liver, and lung.

DNA Preparation. Frozen tissue samples were minced and extracted for total cellular DNA as described previously (20). Briefly, following overnight incubation at 37°C with SDS and protease, the solution was cooled and potassium acetate was added to 1.43 M. After centrifugation, the DNA in the supernatant was precipitated by the addition of ethanol. Precipitated nucleic acids were then treated with boiled RNase A, followed by SDS-protease incubation. Cellular DNA then was extracted with phenol, phenol/chloroform (1/1, v/v), and chloroform, finally concentrated with ethanol, washed with 70% ethanol, and dried in a vacuum.

Agarose Gel Electrophoresis. One-dimensional agarose gel electrophoresis was performed as described previously (21). For discrimination between viral circular and linear episomal DNA, or integrated viral DNA, two-dimensional gel electrophoresis was used (22). Essentially, total cellular DNA is first electrophoresed in the first dimension in a submarine gel apparatus, containing 0.4% agarose in 0.09 M Tris-HCl-0.09 M boric acid-0.003 M EDTA buffer in which was contained 0.5 µg/ml ethidium bromide, for 11 h at 4°C at 50 V. The gel was exposed to UV light and photographed. The lane containing the sample DNA was removed, turned 90 degrees, and laid at the top of a new gel bed. Fresh 1.2% agarose was then poured over the bed and allowed to solidify. The DNA was then electrophoresed in this second dimension 18 h at 24°C at 55 V. A measured grid on a clear plastic film was then placed on the gel and the stained DNA was photographed. The gels were then denatured in alkali and the DNA was transferred to nitrocellulose for hybridization, as described below. In control experiments, the photograped positions of ethidium bromide-stained linear high molecular weight DNA was found to correspond to radiolabeled bands of known integrated HPV DNA and comigrated with cellular DNA when filters were washed and rehybridized with a labeled actin gene probe (data not shown).

Filter Hybridization. Filter hybridization analysis of cellular DNA with or without prior restriction endonuclease treatment was basically as reported previously (20, 21). Following agarose gel electrophoresis, cellular DNA was denatured in alkali, neutralized, and transferred to a nitrocellulose filter by the Southern procedure (23) using a solution of 20X standard saline citrate phosphate (2.4 M sodium chloride-0.3 M sodium citrate-0.27 M potassium phosphate-0.22 M sodium hydroxide). DNA bound to the filter was rinsed briefly in 2X standard saline citrate phosphate and heated at 80°C for 1 h in a vacuum. Filters were preincubated at 37°C for 18 h in 5X Denhardt's solution (24), 0.1% SDS, 20 mM sodium phosphate (pH 6.8), 1 M NaCl, 500 µg/ml depurinated salmon sperm DNA, and 50% formamide. Fresh solution containing 5-10 ng/ml nick-translated (21) HPV DNA probe (specific activity, 2-4 × 10⁸ cpm/µg) and 10% dextran sulfate was added for approximately 18 h at 37°C (75-27°C). HPV 6, 11, 16, and 18 plasmid DNAs were a generous gift from Drs. L. Gissmann and H. zur Hausen. Filters were then washed 3 times at room temperature with 2X standard saline citrate (0.3 M sodium chloride-0.03 M sodium citrate), 0.1% SDS, and 0.1% sodium pyro-

![Fig. 1. Histopathology of perianal tumor and metastatic lesions. Tumors from multiple sites show identical patterns as represented by these examples. A, irregular nests of squamous cells in anal tumor separated by loose myxoid and fibrous stroma. x 100. B, high power view of the tumor nest in liver tumor with prominent koilocytosis (perinuclear clearing and plump hyperchromatic nuclei). Globule of extracellular keratin (center). x 400. C, area with pleomorphic nuclei adjacent to typical koilocytes in liver tumor. x 400. D, immunoperoxidase staining for cytokeratin demonstrates both extracellular and intracytoplasmic deposits of these proteins in liver tumor. x 250.](image-url)
phosphatase, followed by one wash at 40°C for 1 h in 0.4X standard saline citrate-0.1% SDS, and sodium pyrophosphate. Very high stringency washes (Tm-4°C) were performed three times for 1 h each at 65°C using 0.1X standard saline citrate and 0.1% SDS to distinguish between HPV 6 and HPV 11 (25). Dried filters were autoradiographed using Kodak XAR-5 X-ray film (Eastman Kodak, Rochester, NY) and intensifying screens at -70°C.

RESULTS

Hybridization Analysis. Initial analysis of a cutaneous anal primary carcinoma and of lung and liver metastases from this patient under stringent conditions revealed the presence of HPV DNA related to HPV 6 (Fig. 2). The HPV DNA comigrated with high molecular weight cellular DNA in each of the tissue extracts. These data suggested that the HPV DNA was integrated into the cellular genome, although high molecular weight tandem linear viral episomes could not be absolutely ruled out. Very weak bands corresponding to episomal viral DNA were observed in the uncleaved cutaneous anal carcinoma (Fig. 2). These two faint bands appear to be doubly nicked (8 kilobases) and superhelical (apparent size, 6.5 kilobases) viral DNA species. These results were reproducible in separate analyses in which the order of the samples was varied and, thus, are not due to spillover from adjacent lanes. In any event, the presence of episomal viral DNA in this sample is clearly evident in the two-dimensional gel analysis discussed below. When each of these DNA extracts was cleaved with the restriction endonuclease BamHI, the banding patterns for each were virtually identical. Each extract contained a relatively strong band (about 3–10 genome copies/cell) about 8 kilobases long and a band of nearly equal intensity about 6.5 kilobases long, which probably represents a naturally occurring deletion in this HPV genome. We have observed such deletions previously (21), as have other laboratories (26). Also, several weak bands corresponding to about 1 copy/cell of smaller size and two of larger size (10 and 14 kilobases) were observed, which could possibly represent viral-cellular DNA junction fragments.

Further characterization of these HPV species was necessary to confirm the identity of the HPV type and its physical state. Equal amounts of the DNA extracts from each tissue were treated with BamHI, electrophoresed in duplicate, and transferred to filters along with molecularly cloned HPV 6 and HPV 11 DNA standards. Each filter was then hybridized with either HPV 6 or HPV 11 nick-translated DNA probes of equivalent specific activity and then washed under very stringent conditions (Fig. 3). Each of the three extracts hybridized much more strongly to the HPV 11 DNA probe than the HPV 6 DNA probe. Restriction endonuclease analysis of the anal DNA extract using separate or mixed enzymes revealed no cleavage in the 8-kilobase genome with EcoRI, KpnI, or BglII but did detect a single PvuII cleavage site very near the BamHI site and normal HindII, HpaI, AvaI, and PstI cleavage fragments. These data indicate that, with the exception of the loss of a unique KpnI site, this pattern matches that of HPV 11 (27). Together, these data confirm the presence of a HPV 11 variant in these tissues.

Further confirmation of the likelihood of integration of this HPV 11 into the cellular chromosome was obtained through the use of two-dimensional gel electrophoresis. This technique separates viral open circular and superhelical DNA, both unit length and multimers, from linear cellular, nonintegrated viral, and integrated viral DNA (Fig. 4). The cellular DNA extracts from both the lung and liver metastatic carcinomas contained only linear DNA (Fig. 4, the lower of the two possible curves) with the hybridization corresponding to the ethidium bromide-stained high molecular weight cellular DNA observed on the original gel and high molecular weight cellular DNA which hybridized to an actin probe (data not shown). The cutaneous anal primary carcinoma also contained largely linear and high molecular weight linear viral DNA; however, a small amount

\[ \text{Fig. 2. Detection of HPV DNA in malignant tissues from a renal allograft recipient. Total cellular DNA from a primary carcinoma and of lung and liver metastases were electrophoresed in an agarose gel with or without prior BamHI restriction endonuclease treatment. The DNA was then denatured in the gels and transferred to nitrocellulose membranes for hybridization with a labeled HPV 6 DNA probe under stringent conditions which permit the cross-hybridization with HPV 11 but not with other more distantly related HPV DNAs. kb, kilobases.} \]

\[ \text{Fig. 3. Identification of HPV 11 DNA in malignant tissues by very stringent hybridization. Duplicate DNA samples of each of the three malignant tissues were cleaved with BamHI, electrophoresed, and transferred to a nitrocellulose filter. Each half of the filter was hybridized with either labeled HPV 6 (left) or HPV 11 (right) and washed as described in “Patient, Materials, and Methods” at Tm-4°C. Lane 1, normal foreskin; lane 2, anal carcinoma; lane 3, liver metastasis; lane 4, lung metastasis. kb, kilobases.} \]
of episomal viral DNA (Fig. 4D, upper of the two curves) was observed, as expected from the one-dimensional gel electrophoresis described above. This episomal viral DNA might be the result of warty cutaneous precursor lesions adjacent to or intermingled with the malignant tissue. Inasmuch as these experiments were conducted prior to the definitive elucidation of the precise type of HPV DNA present, we used labeled HPV 6 DNA as the probe under conditions where HPV 6 and HPV 11 DNA readily cross-hybridize. Both the one- and two-dimensional gel electrophoresis analyses suggest that the HPV 11 in each of these tumors is largely integrated. We cannot rule out the remote possibility that very high molecular weight multimeric episomal linear viral DNA might exist which comigrates with cellular DNA in a two-dimensional gel analysis. However, we believe that this is very unlikely for two reasons. First, in control studies using condyloma tissue extracts containing very large amounts of episomal viral HPV 6 DNA, by two-dimensional gel electrophoresis we observed only episomal monomers, multimeric circles, and low molecular weight linear viral DNA, which results probably as an artifact of DNA isolation. If even a portion of the multimeric circles were nicked to form multimeric linear viral DNA, we should have observed bands comigrating with high molecular weight cellular DNA, but we did not. Second, while the correlation has not been absolute, it is usually the case that malignant tumors, particularly of the genital tract, and cell lines originating from genital tumors contain predominantly integrated viral genomes of the common, and reputedly more oncogenic, HPV types 16 and 18, as

Fig. 4. Two-dimensional agarose gel electrophoresis of cellular DNA extracts. The use of two-dimensional gel electrophoresis can distinguish between viral episomal circular DNA, linear DNA, and integrated DNA, as described in the text and represented schematically here (4). The results of these analyses on cellular DNA extracts from a primary anal carcinoma (B) and metastatic carcinomas of the lung (C) and liver (D) using a labeled HPV 6 DNA probe under conditions which permit cross-hybridization with HPV 11 are shown (B to D).
well as some other species found less frequently (28, 29). Thus, our detection of HPV 11 as an integrated species in this series of primary and metastatic carcinomas represents a novel finding. Further molecular cloning and detailed characterization of the genome length, subgenomic length, and probable HPV-cellular junction fragments is currently in progress and will be reported elsewhere.

DISCUSSION

Cancers of the anogenital region have been reported to occur with more than a 100-fold greater incidence in patients with renal allografts, as compared with the general population (18). These cancers represent a wide variety of tumor types but include “skin tumors” such as squamous cell carcinoma, non-Hodgkin’s lymphoma, and Kaposi’s sarcoma. Several authors have noted that squamous cell carcinomas of the anogenital region are biologically more aggressive than those occurring in nonimmunosuppressed patients. The term “field effect” aptly applied to the large number of cases which demonstrate that tumors of this area frequently invade local tissue and may metastasize to regional lymph nodes, but rarely disseminate to distant organs (30, 31). The clinical management of these tumors in the immunosuppressed patient is complicated by the fact that they tend to recur after surgical excision and are resistant to chemotherapy. Withdrawal of immunosuppressive therapy, however, has resulted in the cessation of tumor growth and, occasionally, regression of the lesions (32).

This case is a unique example of a highly malignant tumor which developed in a native American male subsequent to receipt of his second allogeneic renal transplant, for which he was treated with immunosuppressive drugs. Histologically, the tumor was a well differentiated squamous cell carcinoma which had arisen in the perianal skin and had been diagnosed as a condyloma acuminatum 2 years previously. The autopsy examination revealed that tumor had substantially replaced the liver, and there were two metastases to the lung. In each site, the tumor was histologically identical and was composed of irregular nests of squamous cells (Fig. 1) and copious amounts of extracellular and intracytoplasmic keratin. Although there was a high degree of nuclear pleomorphism, no cellular inclusions suggestive of virus replication were present and immunohistochemical stains for papillomavirus cytoantigens were uniformly negative, as might be expected in high grade neoplasia.

In situ hybridizations have been used with great efficacy to determine the frequency of human papillomavirus DNA in human precancerous and cancerous tumors, particularly in the anogenital region including the uterine cervix, vagina, and vulvar and penile skin, where up to 58% of the samples have been found to contain HPV DNA (15, 16, 33). Southern blot hybridization is also very sensitive and in a recent study 96% of precancerous cervical tumors were found to contain HPV DNA in amounts inversely proportional to the degree of histological dysplasia (34). Neither of these techniques, however, is capable of determining with certainty the physical state of the viral genome in these tumors.

This report presents data based on Southern blot hybridization and two-dimensional gel electrophoresis to demonstrate that a slightly variant form of HPV 11 DNA was integrated into the cellular genome of a biologically aggressive squamous cell carcinoma in a renal transplant recipient. Integration of viral sequences into the host cellular genome is a widely recognized phenomenon of HPV 16 and 18 DNA, commonly associated with carcinoma of the cervix (35); the observation of HPV 11 DNA integration is unique for this viral subtype, which is largely regarded to be nononcogenic (6, 7). The identical restriction endonuclease cleavage patterns for HPV 11 in all three tissue extracts would indicate that all tumors arose from a single clonal event. Recently, HPV 11 was found in a primary lung carcinoma and metastatic lymph node and hepatic lesions of one patient (36). Most, if not all, of the HPV 11 DNA was episomal, consisting either of normal 8-kilobase genomes or, in the hepatic tissue predominantly an 10- to 11-kilobase episome, which contained a 2.2-kilobase duplication consisting of the 3’ part of the L1 open reading frame, the long control region, and the 5’ part of the E6 open reading frame. While the possibility of an integrated genome existing at low copy number was not ruled out in that report, it appeared that HPV 11 was there also shown to have an oncogenic potential even in the episomal state.

The site of integration of HPV 11 in this case is currently unknown. To date, no preferential site on any chromosome has been noted for other papillomaviruses (37–40). It has been suggested that mutations in the appropriate early HPV genes permit integration at any of several genomic loci, with the concomitant loss of episomal replication (41–43). Disruption of some open reading frames may consequently disengage viral regulation of either its own or the flanking or distal cellular genes, which may be important for cellular proliferation. In this sense, viral sequence integration may represent the initiation step in the multistep process of tumorogenesis (44–47). In contrast to this scheme, mutations in the long control region of HPV 6 DNA may have been sufficient to induce a particularly aggressive verrucous carcinoma without an integration event (48). A similar process involving the duplication of genomic sequences of episomal HPV 11 DNA in a metastatic tumor has been observed (36). The role of HPV in human tumorogenesis appears to be similar regardless of the immunological competence of the host, as reported in the general literature, and, therefore, an explanation of the increased incidence of virally associated malignant tumors in immunosuppressed patients is not directly evident.

Several authors have speculated that infection of a receptive cell by papillomaviruses is by itself insufficient to establish neoplasia but, rather, represents the initiation step of tumorogenesis in the multistep process which ultimately requires promotion of events to maintain deregulated growth (44). Disruption of certain genes, as discussed above, may subsequently alter the regulation of viral or flanking cellular gene products critical to cell proliferation. Recent reports document that the expression of the plasma membrane receptors for such cytokine receptors as epidermal growth factor, interleukin 1, and transferrin, as well as class I HLA antigens, are significantly increased in HPV-infected tissue and cell lines (49–51). These studies add to the growing body of evidence that cytokines may function as tumor promoters in certain environments, such as when the immune system is suppressed. Immunosuppression of the type observed in organ transplant recipients, such as in this case report, results in the selective loss of cytotoxic T-cells, which are reactive to a variety of stimulatory and inhibitory cytokines also known to regulate growth in epithelial cells. In this sense, the resulting imbalance between the growth and inhibition of growth may favor growth in the immunosuppressed patient and, as such, promote tumor progression. Alternatively, this case may exemplify how viral integration alone permitted the evolution of a proliferating clone of epithelial cells and how immunosuppression allowed for an atypical malignant behavior of the tumor. Answers to these questions await the results of molecular cloning and characterization studies now in progress, which may help to identify the key molecular changes required for tumor development.
to transform these viral subtypes into clones capable of integration and cellular oncogenesis. In the interim, however, the growing number of susceptible patients with HPV-associated lesions will require close clinical observation to detect those who will develop highly aggressive tumors, regardless of the type of HPV infection present.

REFERENCES


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