

New Associations of Human Papillomavirus, Simian Virus 40, and Epstein-Barr Virus with Human Cancer

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Approximately 15% of all cancers worldwide appear to be associated with viral infections, and several human DNA viruses are now accepted as causative factors of specific malignancies. Human papillomaviruses (HPVs) cause cervical and anogenital cancers (1). Epstein-Barr virus (EBV) causes infectious mononucleosis and is closely associated with Burkitt's lymphoma, nasopharyngeal carcinoma, and Hodgkin's disease (2,3). HPV is now associated with oral cancers (4–8), EBV with breast and gastric cancers (9,10), and simian virus 40 (SV40) with human mesothelioma and various brain and bone cancers (11–14). Several associations are not universally accepted, and their validity remains controversial. Consequently, the Biological Carcinogenesis Branch of the Division of Cancer Biology at the National Cancer Institute convened a workshop on March 12–13, 2001, in Bethesda, MD, to assess current knowledge concerning associations of HPV, SV40, and EBV with selected human cancers and to define areas where investigation is needed.

HPV and Tumors of the Oropharynx

Head and neck squamous cell carcinomas (HNSCCs) are diseases frequently attributed to environmental factors. Tobacco and alcohol use are well-established risk factors, but HNSCCs also occur in nonsmokers and nondrinkers. Recent epidemiologic and laboratory data suggest that oropharyngeal infection with HPV may predispose to tumorigenesis (15). An association between HPV and some types of HNSCCs has been reported (4–8). HPV16 has been detected in a substantial proportion of squamous cell carcinomas of the soft palate, tonsils, and base of the tongue. HPV16 has been identified in 90% of all HPV-associated HNSCCs and in 50% of all oropharyngeal HNSCCs (4,16). The viral DNA was restricted to the oropharyngeal-tonsillar tumor cells, and viral E6 mRNA was expressed in the same tumors, providing support that HPV16 is involved in that subgroup of HNSCCs (6).

Considerable geographic variation exists in the proportion of oral cancers that are HPV-positive, perhaps reflecting geographic variation in other known risk factors for oral cancers, such as smoking and chewing tobacco, alcohol consumption, sexual behavior, and diet. The natural history of oncogenic HPV infections in the oral cavity is poorly understood. Both sexual and nonsexual transmission of oncogenic HPVs to the oral cavity has been reported. It is unclear why HPV is generally associated with only certain head and neck sites and why HPV16 appears to be the predominate strain associated with head and neck cancers, because other HPV strains with tropism for mucosa, such as HPV18 and HPV31, are also associated with cervical cancer. Different HPV16 strains may be associated with different risks for oral cancer. HPV16 strains have different geographic distributions, and some are more frequently associ-

ated with invasive cervical carcinomas (17–19). Determining the geographic distribution of HPV16 strain variants in HNSCCs would indicate whether similar distributions are associated with oral cancer.

HPV-positive and HPV-negative oral tumors are common and possess distinct features. HPV-positive cancers tend to be classified as nondifferentiated basaloid tumors with wild-type p53 and retinoblastoma protein (Rb) (4,5), tend to have a weaker association with alcohol and tobacco use (in some studies), and tend to have a better prognosis.

Future HPV Research Areas

The lack of a sensitive, validated laboratory test to detect HPV in oropharyngeal tissue and exfoliated cells, the lack of sensitive and specific serologic assays to detect early viral proteins, and the lack of assays to identify the genotype or strain of HPVs in HNSCC tumors and the surrounding nonmalignant tissue impede the design and implementation of laboratory investigations of whether HPV viruses are associated with oral tumors. Cloning the genomes of some of the HPV strains in head and neck tumors may uncover useful information concerning strain variants in HNSCCs. HPV16 variants carrying different viral coat proteins could be examined for their ability to infect different epithelial types, viral receptors on various epithelial tissues could be characterized, and viral gene transcription could be examined in HPV-positive oral cancer and precursor lesions. Molecular biologic and genetic studies involving the use of gene array technology are needed to elucidate cellular gene expression patterns associated with pathologic changes. Other studies are needed to clarify whether the HPV genome is integrated in HNSCC tumor DNA and whether tumor cells and their associated HPVs are monoclonal, to determine the molecular pathways involved in the development of oral cancer, and to identify the cofactors necessary for initiation, promotion, and progression to malignancy. Results in HNSCCs can then be compared with results from cervical and anogenital cancer.

If viral gene expression and effects on cultured oral epithelial cells are similar to results in cultured cervical or foreskin epi-

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thelium, then HPV gene activity should be examined in more physiologic situations, such as in tissue sections and animal models that more closely resemble the human disease. Such a system is the K14-HPV16 transgenic model (mice in which the HPV16 E6/E7 gene is expressed in the epidermis by the human keratin 14 promoter) that produces oral hyperplasia (20,21). HPV-positive oral cancers presumably arise from HPV-induced precursor lesions as in cervical cancer, but further characterization of the events between the primary HPV infection and subsequent oral cancer is needed.

HPV testing could be evaluated as a method of early detection and as a factor in treatment and management decisions for HPV-positive and HPV-negative cancers and for monitoring relapse of HPV-positive cancers after treatment. Vaccines for anogenital HPV-associated cancers and premalignant lesions and for genital tract infection by HPV16 and other oncogenic HPVs are currently being developed and tested. Promising candidate vaccines should be considered for clinical trials targeting HPV-associated oral cancers. HPV vaccines active against cervical HPV infections might also prevent oral HPV infections, but genital HPV vaccines might not be given until just before puberty and would not protect against infection acquired earlier. This possibility underscores the need for information about the natural history of HPV-associated oral cancers. Additionally, although the role of HPV in nonmelanoma skin cancers was not specifically addressed in formal presentations, general discussion suggested that some recent studies (22–24) had demonstrated a presumptive association that should be explored further.

SV40 in the Human Population

The origin of SV40 in the human population is unknown. SV40 was a contaminant of the Salk and Sabin poliovirus vaccines derived from the rhesus monkey kidney cells that were used to manufacture the vaccines. There is serologic evidence that SV40 infection may have antedated the contaminated poliovirus vaccines (12,14,25,26); however, the antibodies were not distinguished from those produced by the related papovaviruses, JC and BK. SV40 does not cause symptoms or disease in monkeys but does transform cultured human cells and does produce tumors when inoculated into newborn hamsters. The prevalence and distribution of SV40 infection in the human population is a subject of continuing research and some controversy.

Detection of SV40 in Human Cancers

SV40 DNA has been sporadically detected in human tumors. SV40 has been detected predominantly in samples from mesotheliomas, brain tumors, and osteosarcomas (11–13,27) and has been detected most frequently in mesotheliomas, choroid plexus tumors, and ependymomas, although with wide variations. SV40 DNA was detected with polymerase chain reaction (PCR) assays using primer sets directed against one or more regions of the SV40 genome, followed sometimes by Southern blot analysis or sequencing of PCR products. SV40 T antigen was detected by immunohistochemistry or western blot analysis. SV40 mRNA was detected by *in situ* cytohybridization, and infectious SV40 was isolated from some tumor samples (12,14). In a multi-institutional study (28), several participating groups showed the reproducibility of detecting SV40 DNA sequences in mesothelioma specimens. The diversity of sequenced PCR-amplified sequences ruled out contamination with common laboratory

strains of SV40 (29). The regulatory region of SV40 DNA detected in tumors often had the archetypal arrangement of natural isolates rather than the duplicated enhancer arrangement of laboratory strains. Additional nucleotide differences occur in the carboxyl terminus of the T antigen, a variable region differing in laboratory strains and natural isolates (12). Consequently, the sequence differences detected may not be caused by laboratory contamination or nucleotide substitutions introduced during PCR amplification. Importantly, when mesothelioma samples were microdissected, SV40 DNA was detected by PCR in tumor cells but not in adjacent nonmalignant cells (30).

SV40 T antigen is the major regulatory oncoprotein and is involved in viral DNA replication, transcriptional control, virion assembly, and transformation. T antigen is thought to induce transformation by interacting with key cellular proteins that normally function to regulate cell proliferation. T antigen expression was detected immunohistochemically in tumor cells, and T antigen protein isolated from some human tumor specimens can form a complex with p53 or Rb (25,31), which would inactivate these tumor suppressor proteins. If such complexes are disrupted, the transformed phenotype of tumor cells should be reversed. Finally, when an adenovirus vector was used to express antisense SV40 early-region transcripts (which bind to the corresponding T antigen sense transcripts) in human pleural mesothelioma cell lines, growth was inhibited, and SV40 DNA-positive cells, but not SV40 DNA-negative cells, were induced to undergo apoptosis (32).

Cultured human mesothelial cells are efficiently infected with and transformed by SV40 (33), and asbestos, a risk factor for mesothelioma, increases the transformation frequency. In some cultures derived from SV40-positive mesotheliomas, maintenance of the transformed phenotype depends on continuous T antigen expression.

Validity of Detection of SV40 in Human Cancers

Many epidemiologists and some laboratory investigators remain unconvinced of the validity or consistency of detection of SV40 in human tumors or the significance of the various reported detections. PCR findings of SV40 vary from laboratory to laboratory and require a high number of PCR cycles to obtain a positive result. In those investigations using only PCR evidence, the high number of PCR cycles needed could reflect a low virus copy number, the possibility that SV40 is present in only some of the tumor cells, and the presence of stromal tissue. Laboratory contamination has been suspected, but viral sequence data weigh against this concern. Such results emphasize the need for corroborating evidence of SV40 in tumors, which has been obtained in several studies. The serologic evidence for SV40 infection of humans remains unclear. Epidemiologic studies based on serology also have yet to demonstrate clearly that SV40 circulates in the human population or a clear association between exposure to SV40-contaminated poliovirus vaccine and increased tumor incidence. However, the studies done to date do not exclude these possibilities. Because SV40 can transform human cells, additional research and coordinated studies, including new epidemiologic studies that make use of virus-specific markers and are targeted to the malignancies that have been implicated, are needed to clarify these complex issues.

Research Opportunities

Coordinated efforts are needed to obtain tumor specimens collected at biopsy or by excision from patients with brain, bone,

and mesothelial tumors to determine whether SV40 DNA and proteins are present. Identified SV40 DNA should be characterized by determining whether the SV40 DNA is episomal or integrated, which regions of the viral genome are present, and whether mutations and DNA sequence divergence occur in the oncoprotein coding and transcriptional control regions. Relations should be explored between specific tumor types, the status of the viral genome, and the expression of large T and small T antigens. Well characterized monoclonal antibodies that distinguish SV40 T antigen from related antigens in BK virus and JC virus should be developed and used for immunocytochemistry. Transforming proteins, the expression of viral genes as mRNAs and proteins, the interaction of T antigen with tumor suppressor proteins, and the status of cellular p16/p19ARF, Rb, and p53 pathways should be characterized.

Transgenic mice have been used to document the antigenicity of SV40 T-antigen-transformed cells and to show that a T-lymphocyte-mediated immune response can control the growth of primary tumors *in vivo*. T-antigen-specific epitopes have been identified that can be presented by human major histocompatibility complex class I molecules. In one instance, epitope-specific T cells have been identified in HLA-matched mesothelioma patients. These promising results need to be extended to define HLA-restricted SV40 T-antigen-specific cytotoxic epitopes that can be used to monitor patients for T-antigen-specific T cells by use of a tetramer analysis, to develop immunogenic expression systems for use in immunotherapy protocols, and to measure levels of SV40-specific antibodies in patients over time.

EBV and Human Cancers

EBV is a herpesvirus that is endemic in the human population, with more than 90% of normal adults worldwide being seropositive. EBV is the primary etiologic agent for infectious mononucleosis, post-transplant lymphoma, and oral hairy leukoplakia. Most adults are symptomless healthy carriers. However, EBV is strongly associated with Burkitt's lymphoma, nasopharyngeal carcinoma, undifferentiated parotid carcinoma, a subset of patients with Hodgkin's disease, and a variety of B-cell lymphoma (2). EBV has also been associated with undifferentiated gastric carcinomas, about 10% of which worldwide contain EBV DNA and proteins (34) and, most recently, with aggressive human breast cancers (9).

In cancers with a strong EBV association, EBV DNA and proteins are detected in all tumor cells (3). The EBV genome is clonal (i.e., developed from a single EBV genome, as demonstrated by the homogeneous number of terminal repeat elements) in Burkitt's lymphoma, Hodgkin's disease, nasopharyngeal carcinoma, parotid tumors, and gastric carcinomas (35–37). Different sets of viral proteins are expressed in different EBV-associated tumors. Burkitt's lymphomas and gastric carcinomas express EBV nuclear antigen 1 (EBNA1) protein; Hodgkin's disease tumors and nasopharyngeal carcinomas express EBNA1, EBV latent membrane proteins LMP1 and LMP2, and transcripts encoded by the EBV *Bam*HI A fragment (2,38). Lymphomas that develop in immunosuppressed patients after organ or bone marrow transplantation express all EBV proteins detected in latently infected B lymphocytes *in vitro*, namely EBNA1, LMP1, LMP2, LMP2B, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP proteins, the latter EBNA3s being major targets for cytotoxic T lymphocytes (37). In most EBV-associated diseases, EBERs (abundant nonpolyadenylated

RNAs) are expressed. However, EBERs are not expressed in regions of differentiated epithelial cells in nasopharyngeal carcinoma and also in the permissive infection of oral hairy leukoplakia (39,40).

Detection of EBV in Breast Cancer

Data linking EBV to a subset of breast cancers include detection of low levels of EBV DNA by real-time PCR and Southern blot analysis and the immunohistochemical detection of EBNA1 (9). EBV has been detected more frequently in aggressive breast tumors than in nonaggressive tumors; approximately 70% of advanced breast cancer tumors are EBV-positive (9). Estrogen receptor-negative tumors are more likely to be EBV-positive than EBV-negative; 80% of EBV-positive tumors are estrogen receptor-negative (9). The frequency of EBV-positive tumors is also higher in breast cancer patients with three or more metastatic lymph nodes than in patients with fewer metastatic lymph nodes (9). EBV DNA was detected in 31.8% (162) of 509 primary invasive ductal breast cancer samples from geographic areas with various risks for nasopharyngeal carcinoma (41). Laser capture microdissection combined with real-time quantitative PCR showed that EBV DNA was present in malignant epithelial cells but not in normal stromal cells.

Other investigators (42–45) have screened breast cancer samples for EBV EBER expression but did not detect it. Investigators (43,45) using EBER *in situ* hybridization and EBNA2 and LMP1 immunohistochemistry and probing for transcripts of EBNA1 and EBERs failed to detect EBV. However, Chu et al. (46) identified EBER1-positive breast carcinoma cells in five (10%) of 48 samples, but only a few cells in these samples were EBV-positive. Southern blot analysis did not detect EBV. This observation should be compared with other EBV-associated malignancies, noted above, in which virtually all neoplastic cells were EBV-positive. Because EBER expression was not detected in EBNA1-positive breast cancer samples, the failure to detect EBV in breast cancer samples may reflect a failure of these tumors to express EBERs or to express them at a detectable level. Multiple EBV assays should be applied to these tumor samples to clarify these observations.

The association between EBV and breast cancer was also explored in a nude mouse model developed to investigate aggressive breast cancers (9). Human breast cancer cells infected with EBV *in vitro* express EBERs, EBNA1, and LMP1. When these cells were injected into nude mice, tumors developed in all mice given cells that expressed LMP1, EBERs, and EBNA1. Uninfected breast cancer cells produced tumors in the mice, and the presence of EBV did not appear to affect the growth rate of the tumors. This mouse model may be useful in developing therapies for EBV-positive cells. Other model systems could be used to investigate mechanisms of EBV latency and gene expression in breast cancer.

EBV Infection of Epithelial Cells

Latent EBV is consistently detected in nasopharyngeal carcinoma but has not been detected in normal epithelial cells. However, permissive infection in oropharyngeal epithelial cells has been reported (47). An environmental or genetic cofactor may allow EBV to establish a latent infection and express viral transforming proteins. Although latent or replicative EBV has not been detected in normal breast epithelium, EBV has been detected in breast milk, which might increase the chance of

EBV entering a breast epithelial cell and establishing a latent infection.

Distinct cellular receptors are probably required for EBV to enter cells. For example, the entry of EBV into B cells first requires that the viral envelop glycoprotein gp350/220 bind to the cellular complement receptor CD21 or CR2 (48,49) and then requires that the viral glycoprotein gp42 bind to a co-receptor, in this case, an HLA class II molecule. CD21 and HLA class II molecules contribute to a high-efficiency EBV infection. However, EBV can infect epithelial cells independently of whether the cells express CD21 and HLA class II molecules, and it has been suggested that cell-to-cell contact may be a mechanism for efficient infection of epithelial cells by EBV (50–52). Close cell-to-cell contact may facilitate the accessibility of EBV to a novel low-affinity receptor on the target epithelial cells. It is possible that EBV enters breast epithelium by cell-to-cell contact.

High-Priority EBV Studies

The first priority should be more extensive screening of breast cancer samples for EBV, with such methods as laser microdissection, quantitative PCR, *in situ* EBV DNA hybridization, immunohistochemistry, and real-time PCR, to investigate an association between the extent and distribution of multiple EBV mRNAs, viral load, and EBV-positive breast cancers. EBV-positive breast cancer tissues should be characterized to determine the type of latency and to identify which viral genes and serologic markers are expressed for subsequent epidemiologic screening.

Aggressive breast cancers that develop in younger women who do not carry a BRCA1 mutation should be carefully examined for the presence of EBV DNA or virus-specific proteins. These cancers do not appear to be hormonally associated and thus may be associated with EBV. It should be determined whether EBV is associated with breast cancer as a primary etiologic factor or as a cofactor that contributes to malignant or invasive potential (53,54).

CONCLUSION

More research is needed to resolve the controversial issues of whether HPV is involved in nonmelanoma skin cancer, whether SV40 is involved in human cancers, and whether EBV is involved in human breast cancer. Recent reports that several cancer-causing viruses may be implicated in other cancers suggest that these and other viruses could be involved in additional human cancers. General methods to determine whether infectious agents cause specific diseases have been addressed elsewhere (55,56), but new approaches need to be developed to comprehensively determine whether known and as yet unknown viruses and microbial agents are associated with human cancers. The public health consequences that may result from these studies could reduce the cancer burden worldwide.

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NOTES

The overall workshop was chaired by Dr. Joseph Pagano (University of North Carolina) and co-chaired by Dr. John Schiller (National Cancer Institute) for presentations on HPV; Dr. Satvir Tevethia (Pennsylvania State University at Hershey) for discussions on SV40; and Dr. Nancy Raab-Traub (University of North Carolina) for new disease associations with EBV. In addition to the authors, the participants at this meeting were as follows: Richard Ambinder, Janet Butel, Michele Carbone, Joakim Dillner, Denise Galloway, Robert Garcea, Maura Gillison, Diane Hayward, Rolando Herrero, Michael Imperial, Irene Joab, Kamel Khalili, Jeffrey Kopp, Barbara Krynska, Paul Lambert, John Lednický, Andrew Lewis, Richard Longnecker, Eugene Major, Nancy Mueller, Karl Munger, Harvey Pass, Keith Peden, David Schrupp, Keerti Shah, Elaine Smith, Bill Sugden, Mary J. Tevethia, and Lubomir Turek.

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