Molecular Diagnosis of Infection-Related Cancers in Dermatopathology

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The association between viruses and skin cancer is increasingly recognized in a number of neoplasms, that is, cutaneous squamous cell carcinoma, Kaposi sarcoma, nasopharyngeal carcinoma, and Merkel cell carcinoma, as well as hematolymphoid malignancies such as adult T-cell leukemia/lymphoma and NK/T-cell lymphoma (nasal type) and post-transplant lymphoproliferative disorders. Molecular assays are increasingly used to diagnose and manage these diseases. In this review, molecular features of tumor viruses and related host responses are explored. The tests used to identify such features are summarized. Evaluation of the utility of these assays for diagnosis and/or management of specific tumor types is presented.
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In the United States, approximately 10% of all cancers are thought to be related to infection. Worldwide, infectious agents are associated with 20% of all cancers.[1] Most of the implicated microorganisms that are recognized in this role are viruses, commonly referred to as "tumor viruses." A smaller number of cancers may be associated with bacterial infection.

Viral infection is thought to initiate tumor transformation. The insertion of viral genes into human DNA may mutate or activate oncogenes and allow unregulated cellular proliferation.[2] Virus-related inflammation may lead to genomic instability and oncogenic mutagenesis.[3] The skin is an ideal site for the development of infection-related cancer, owing to the confluence of viral exposure, ultraviolet irradiation, and immunologic susceptibility.[2] In skin cancers, known tumor virus families include Retroviridae, Polyomaviridae, Herpesviridae, and Papillomaviridae. The International Agency for Research on Cancer accepts 7 human viruses as group 1 (carcinogenic to humans), 5 of which are associated with skin cancers, including Epstein–Barr virus (EBV), human papillomavirus (HPV), human T-cell lymphotropic virus type 1 (HTLV-1), human herpesvirus 8 (HHV-8), a Kaposi sarcoma-associated herpes virus (KSHV), and human immunodeficiency virus (HIV) type 1.[4]

As human tumor viruses are prevalent, and as virus-driven neoplasms are increasingly identified with advancing technology, it is important to know how to use relevant molecular tests to gain meaningful diagnostic, prognostic, and therapeutic information for individual patients. The purpose of this review is to summarize the role of individual tumor viruses in skin cancers, and to discuss the molecular-based tests that are used to identify these viruses and associated features (Table 1). Finally, we will comment on the utility of key tests in cancer diagnosis and therapy (Table 2).

Papillomaviridae (DNA Virus, HPV)

HPVs are small double-stranded DNA viruses composed of viral genomic DNA and coat.[5] The viral genome encodes 8 proteins: E1, E2, E4, E5, E6, E7, L1, and L2. Of these, L1 and L2 are envelope proteins. E5, E6, and E7 are involved in cell proliferation and survival. High-risk HPV subtypes such as 16 and 18 are thought to play a role in malignant transformation of cells by producing E6 and E7 viral regulatory proteins.[6] In these HPV types, E6 may interact with and degrade the p53 tumor suppressor gene,[1,7] whereas E7 binds the retinoblastoma (Rb) tumor suppressor gene, resulting in deregulated cell proliferation and transformation.[8]

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Table 1 Select Molecular Techniques Used in Analysis of Infection-Related Skin Tumors

Technique	Principle	Uses and Advantages	Disadvantages
IHC, IF	Ab:Ag reaction on a glass slide. Chromagen-linked second Ab and light microscopy (IHC) or fluorescent-labeled Ab and fluorescent microscope (IF)	Identifies proteins; automated, high-throughput; semiquantitative; paraffin (IHC) or frozen tissue	Not useful with poorly expressed Ag; not helpful with nonspecific Ab; requires validation with each new Ab; needs appropriate controls
WB	Ab:Ag reaction on resolved tumor protein extracts. Chromagen or enzyme-linked second Ab; signal is colorimetric or chemiluminescent	Identifies protein; used for confirmation of Ab screening tests; high specificity	Labor-intensive; requires skilled technical staff; time-consuming
SB	Nucleic acid probes hybridize digested, size-separated DNA. Fluorescent tags or chromogenic dyes on bound probe produce a signal	Identifies DNA sequences; can distinguish integrated vs episomal virus	Labor-intensive; requires skilled technical staff; time-consuming; requires large amounts of DNA; cannot be used on FFPE
ELISA	Ag:Ab reaction on a solid support with patient sera; enzyme-linked Ab or Ag, detected by color, fluorescence, or electrochemiluminescence	Identifies proteins in serum; good for screening large populations; high sensitivity; numerous available kits	Kits have different combinations of antigenic source, solid phase, and type of assay, resulting in range of sensitivity/specificity; low specificity
ISH, FISH, CISH	RNA or DNA probes hybridize with target nucleic acid sequences on glass slide or tissue microarray. Fluorescent tags (FISH) or chromogenic dyes (CISH) on the bound probe; light or fluorescent microscopy combined with digital analysis	Identifies nucleic acids in tumor cells or tissue; high specificity; high resolution; can use multiple probes and fluorochromes in same test, to assess signal ratios; immuno-FISH can identify cell types being labeled; can distinguish episomal vs integrated virus	Requires skilled technical and interpretive skills
PCR, RT-PCR	DNA and RNA fragments are amplified with PCR primers, DNA polymerase, ± reverse transcriptase, and nucleic acids; PCR products are analyzed by RFLP and agarose gel electrophoresis, or DNA probes, assayed on an oligonucleotide microarray, or by direct sequencing	Identifies nucleic acid sequences; high sensitivity; little DNA needed; specificity of primers; multiplex primers; can be quantitative (real-time PCR); microarray analysis can identify multiple synchronous infections; can use FFPE	Amplification of a nonspecific product (false-positive), and, rarely, false-negatives; dependent on quality of template DNA or RNA; microarray requires complex expensive analytical equipment; cannot distinguish episomal vs integrated virus; cannot distinguish tumor cells vs microenvironment
DNA microarray	DNA is reverse-transcribed from RNA with fluorescent tags and hybridized to DNA sequences on solid microarray	Detects differences in gene expression profiles; can examine large numbers of DNA sequences; nonamplified product may have fewer false-positives	Fresh tissue is needed; depends on purity of cell populations; requires significant skill and labor; expensive

Ab, antibody; Ag, antigen; IHC, immunohistochemistry; IF, immunofluorescence; WB, Western blot; SB, Southern blot; FFPE, formalin-fixed paraffin-embedded tissue; ELISA, enzyme-linked immunosorbent assay; ISH, in situ hybridization; FISH, fluorescence in situ hybridization; CISH, chromagen in situ hybridization; PCR, polymerase chain reaction; CGH, comparative genomic hybridization; RFLPL, restriction fragment length polymorphism.

HPV infection typically undergoes an active phase, after which most people mount an effective immune response, become DNA-negative, and develop antibodies to the L1 protein over several months. Of these patients, 10%-20% will remain DNA-positive.[9]

There are > 120 genotypes of HPV recognized, of which at least 15 are thought to be oncogenic. The recent consensus by the working group on the taxonomy of papillomaviruses and the International Council on Taxonomy of Viruses defines an HPV type as a complete genome, whose L1 gene sequence is at least 10% different from any other HPV type.[10] According to the meeting of the International Agency for Research on Cancer in 2009, HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 59 are considered carcinogenic, and 68 is "probably" carcinogenic.[4,11]

HPV in Cancer

HPVs have been divided into mucosal or skin types. The term "high-risk" HPV has historically signified HPV subtypes strongly associated with the development of urogenital and oral squamous intraepithelial neoplasia and carcinoma. High-risk HPV subtypes 16 and 18 are responsible for up to 70% of all cervical cancers.[12] Low-risk HPVs are found within warts of oral and urogenital mucosa, and have not been shown to have a significant association with carcinoma.

In 2004, a phylogenetic classification was adopted by the International Council on Taxonomy of Viruses, replacing the categorization of HPV types into mucosal or skin types, or high- and low-risk types, with a Greek letter designating a genus.[10] Alpha genus (alphapapillomaviruses) includes most high-risk HPV and many low-risk HPV related to benign skin lesions. Beta genus (betapapillomaviruses) represents HPV related to epidermodysplasia verruciformis (EV, see later in the text), transplant recipients, and, increasingly, cutaneous squamous cell carcinoma (cSCC).

In the skin, HPV localizes to the epidermis, producing common flat and plantar warts in immunocompetent individuals. Studies have identified 3 cSCC types that are disproportionately associated with high-risk (alpha genus) HPV.[13] These include periungual/digital cSCC, bowenoid papulosis, and, possibly,

Table 2 Suggested Clinically Useful Molecular Tests in Evaluation of Infection-Related Skin Neoplasms

Neoplasm	Assay(s)	Target Molecule	Utility
cSCC	ISH	High-risk/alpha HPV	Determine extent of therapy in digital cSCC and predict recurrence
	IHC	P16 protein	Determine extent of therapy in digital cSCC and predict recurrence
ATLL	ELISA	EBV virus lysate, or synthetic peptide	Screening and diagnosis*
	WB or RT-PCR	Gag/p24, env gp46, gp61/68	Screening and diagnosis; RT-PCR can distinguish HTLV subtype and quantify VL
NPC	ELISA PCR/RT-PCR	EA, EBNA1/VCA-p18 EBV-DNA Carcinoma-specific BARF1 mRNA	Screening Screening and diagnosis; Can use nasopharyngeal brush or plasma; Confirm positive ELISA; Southeast Asian and Chinese populations
	ISH	EBV-encoded RNAs	Diagnosis, any population
NKTCL	EBER-ISH Real time PCR	EBV-encoded RNAs EBV BamHI-W region	Diagnosis, any population Pretreatment prognosis from plasma VL; Stratify for therapy
PTLD	RT-PCR (serial)	EBV-DNA in plasma	Estimate risk of PTLD: may result in decreased immunosuppression or addition of rituximab; Monitor disease activity
	EBER-ISH	EBV-encoded RNA	Diagnosis
DLBCL-E	EBER-ISH	EBV-encoded RNA	Diagnosis
KS	LANA-1 IHC or ISH	HHV-8 LANA/ORF73	Diagnosis
MCC	CM2B4 ELISA	LTA LTA > VP1	Diagnosis in difficult cases Pending validation for monitoring disease

cSCC, cutaneous squamous cell carcinoma; ISH, in situ hybridization; HPV, human papillomavirus; IHC, immunohistochemistry; ATLL, adult T-cell leukemia/lymphoma; ELISA, enzyme-linked immunosorbent assay; WB, Western blot; PCR, polymerase chain reaction; NPC, nasopharyngeal carcinoma; NKTCL, NK/T-cell lymphoma, nasal type; RNA, ribonucleic acid; PTLD, post-transplant lymphoproliferative disorder; VL, viral load; DLBCL-E, diffuse large B cell lymphoma of the elderly; KS, Kaposi sarcoma; HHV-8, human herpesvirus 8; ORF, open reading frame; MCC, Merkel cell carcinoma; LTA, large T-antigen.
*Consider more than 1 ELISA in endemic populations.

verrucous carcinoma. In an analysis of 103 patients with HPV-associated cSCC of the digits, the most commonly identified HPV was HPV16, found in 74% of cases. HPV-associated cSCC versus non-HPV-associated cSCC was more difficult to eradicate and exhibited a higher recurrence rate (20% vs 3%), but showed no difference in metastatic rate.[14] As in cervical carcinoma, p16 expression, which relates to viral E7 protein/Rb interactions, may be increased in HPV-associated digital cSCC versus non-HPV-related digital cSCC, supporting p16 as a surrogate marker for HPV.[15]

HPV-related cSCC not associated with high-risk HPV, and often of the beta genus, is increased in 3 populations: patients with EV, an inherited disorder of reduced immunity resulting in multiple wart-like lesions; transplant recipients; and HIV patients.[16] Of patients with EV, 50% will develop cSCC.[17] Patients with EV are predisposed to infection with HPV types 5, 8, 9, 12, 14, 15, 17, 19-25, 36, 38, 47, and 50 ("EV-HPV")[18,19]; HPV types 5 and 8 are associated with >90% of EV-associated cSCC.[20] EV-HPV types are associated with cSCC within the transplant population.[21]

Viral oncogenesis in EV-HPV related–neoplasia appears to differ from that in high-risk HPV. The E6 protein of EV-HPV types 5 and 8 does not bind cellular p53, or promote p53 deg-radation. EV-HPV E7 proteins do not interact with pRb.[18] E6 proteins of HPV types 5, 10, and 77 may target cellular Bak protein for proteolysis, potentially inhibiting apoptosis, and causing tumors, especially in the context of ultraviolet-induced damage.[22] Predictably, p16 expression is not elevated in these tumors.[23]

As polymerase chain reaction (PCR) techniques improve, the identification of HPV DNA in immunocompetent patients' cSCC has increased, to include as many as 60% of lesions tested.[16,24] Studies show an increased risk of cSCC in immunocompetent patients with HPV+ hair follicles, which may be a reservoir for the virus, and/or positive antibodies, and includes patients whose baseline sera were drawn 18 years before development of cSCC.[25] The risk for cSCC increases with positive antibodies to multiple HPV types,[26] with antibodies to EV-HPV types 5 and 76, and/or on sun-exposed skin.[25,26] Persistence of DNA in hair follicles and persistent antibodies over time appear to increase the risk of cSCC.[25]

Differing human leukocyte antigen (HLA) haplotypes may alter the risk for cSCC, particularly in transplant populations,[27] where they may indicate differing immune responses to the virus.
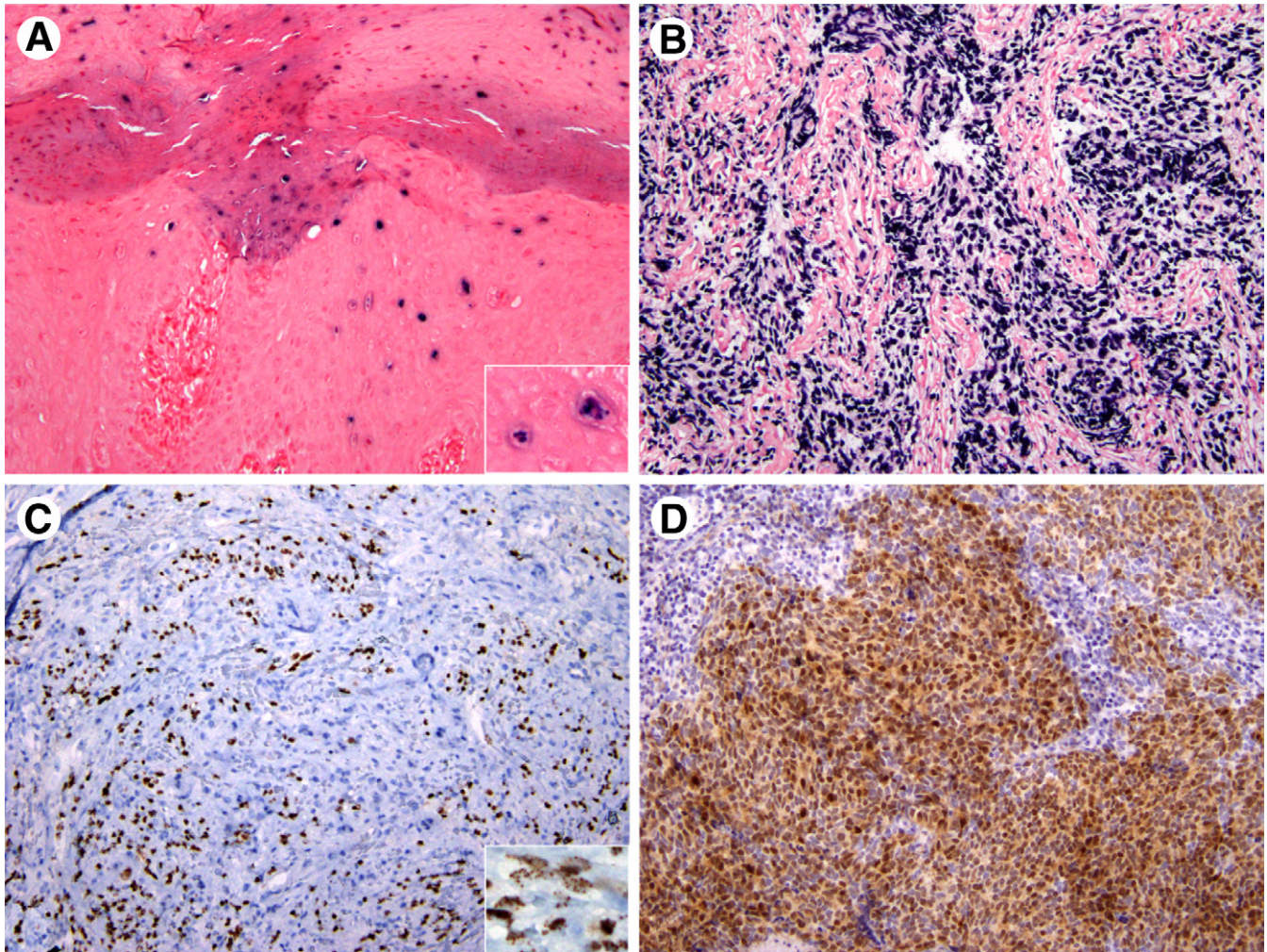
Figure 1 Immunohistochemistry and in situ hybridization studies. (A) INFORM HPV III FAMILY probe (Ventana, Tucson, AZ) showing both globular (episomal) and punctate (inset, integrated) human papillomavirus in a digital squamous cell carcinoma. (B) Epstein–Barr virus-encoded RNA (EBER) labels nuclei of epithelial cells in nasopharyngeal carcinoma. (C) LANA-1 immunohistochemistry shows a punctate pattern (inset) of human herpesvirus 8 viral proteins. (D) Monoclonal antibody 2B4 labels the nuclei of Merkel cell carcinoma.

Molecular Testing for HPV-Related Skin Cancer

Molecular assays available for the detection of HPV (Table 1) have been well developed for cervical/anogenital tumors, but less so for skin.[5] Regarding immunohistochemistry (IHC), antibodies against p16, E6, and E7 require further analysis in cSCC, and are not used.

Direct hybridization assays used for HPV testing include Southern blot (SB) and in situ hybridization (ISH). SB is impractical clinically, given the labor, time, and quantities of DNA required. Although HPV probes targeting various HPV types are commercially available, the specificity of ISH is lacking, ranging from 30% to 72% in carcinoma and condylomatous lesions.[28] Chromagen (ISH) panels with higher specificity may suffer lower sensitivities.[29] In appropriate lesions, ISH may distinguish episomal versus integrated virus; ISH for HPV-16 E6 stains diffusely if episomal and punctate if integrated (Fig. 1A).[30] However, commercial ISH panels do not typically target betapapillomavirus.

Signal amplification hybridization assays for HPV used in cervical cancer screening use proprietary cocktails of DNA or RNA oligonucleotide probes for hybridization, followed by chemiluminescent or amplified fluorescent signal reactions. These are limited by their inability to discriminate between HPV types, and are designed for examination of cervical lesions[31] with brush/broom methodology and are generally limited to the detection of alphapapillomaviruses.

Target amplification assays include real-time polymerase chain reaction (RT-PCR), which can quantify small amounts of HPV DNA, provide a measure of viral load (VL), and distinguish episomal HPV from integrated HPV.[32] High-throughput assays using multiplex RT-PCR can simultaneously detect high- and low-risk HPV and controls in the same reaction.[33] RT-PCR for the identification of E7 transcripts may have clinical utility in cervical disease,[34] but its utility is unclear in cSCC. Custom multiplex assays for betapapillomavirus are used primarily in research.

At this time, there are no indications supporting the use of

ISH or signal and target amplification tests in the diagnosis of most cSCC. ISH for alphapapillomaviruses might have use in some digital cSCC in terms of predicting recurrence and planning therapy. ISH HPV positivity or high HPV VLs are commonly found in subpopulations such as transplant patients.[35] HPV is also seen in non-neoplastic hyperproliferative disorders such as psoriasis in this population, and is not diagnostic of neoplasia. Furthermore, although the detection of surrogate markers such as p16 or E7 transcripts is useful in cervical and anogenital biopsies, the use of such tests should remain limited to digital cSCC, in planning therapeutic interventions. Anti-HPV serologic tests remain outside the spectrum of clinical utility.

Retroviridae (RNA Virus, HTLV-1, HIV)

Worldwide, 15-20 million people are infected by HTLV-1, with endemic populations in the Caribbean and Japan. Of HTLV-1 carriers, 5% will develop adult T-cell leukemia/lymphoma (ATLL),[1] and 50% of these will develop skin involvement.[17]

HTLV-1 is a single-stranded RNA virus with a diploid genome, reverse transcribed to proviral DNA, which randomly integrates into the host cell genome, typically within CD4+ T-cells.[36] Early on, HTLV-1 spreads by cell-to-cell transmission, through an extracellular "viral synapse."[37] As the disease becomes chronic, the virus survives through increased T-cell proliferation, with persistent clonal proliferation of infected cells in the absence of malignant disease.[38]

The HTLV provirus contains gag, pol, and env genes, flanked by a long terminal repeat (LTR). HTLV-1 contains a unique component, pX, located between the env and 3′-LTR, which encodes viral regulatory and accessory proteins Tax, Rex, p8, p12, p13, p21, p30, and the HTLV-1 basic leucine zipper factor (HBZ).[1,39] Tax is thought to be a key oncogenic protein in HTLV-1, which can enhance or repress transcription of viral and cellular gene products, through interactions with nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB)/p53, tumor necrosis factor beta (TNF-beta), interleukin-2/interleukin-2 receptor, cyclin-dependant kinase 4, and Rb, interfering with DNA repair, and deregulating proliferation.[1,39,40] HBZ may play a role in leukemogenesis through interactions with Tax and NF-κB/p65.[39,41] Tax is present only in 40% of ATLL cases, possibly owing to transcriptional silencing.[42] HBZ is present in all ATLL cases.[43]

Active HTLV-1 infection is typically followed by a host immune response, antibody production, and cytotoxic T-cell targeting of viral antigens, with subclinical chronic infection persisting in most patients. Early cytotoxic T-cell responses appear to target the envelope surface unit glycoprotein 46 (gp46).[44] A subset of patients do not seroconvert and are able to clear the virus with the cell-mediated response.[45] ATLL latency is years to decades; early exposure is associated with a greater risk of malignant disease.

HTLV-1 in Cancer

In HTLV-1 infection and ATLL, clonality of T-cell populations can be measured by consistency of proviral insertion sites or specificity of T-cell receptor gene rearrangements.[1] Viral integration appears to be random and inconsistent during early infection, and may result in the finding of numerous clones in a single blood sample. Some clones may persist over time, with fluctuation in the number of cells suggesting a successful host immune response. Patients with asymptomatic infection may possess high pro-VLs measured by RT-PCR or SB,[46] reflecting the accumulation of multiple proliferating persistent clones, rather than neoplastic disease. High VL with oligoclonal bands may be a risk factor for the development of ATLL.[46]

In oligoclonal proliferations of ATLL, HTLV-1 provirus integrates with 1 or 2 copies at the same chromosomal locations in each cell, often close to transcriptional start sites of cellular genes.[36] Monoclonal or oligoclonal integration patterns may be seen in leukemic cells[47] or different integration sites within peripheral blood cells versus affected skin,[48] indicating the presence of more than 1 clonally expanded infected cell. One study showed no clinical difference between patients with monoclonal or polyclonal bands or smears on SB, in the smoldering variant of ATLL. However, CD4+ counts appeared to increase with decreasing clones, and antibodies such as CD25 and CD4 and the CD26/CD25 ratio appeared to predict a monoclonal band.[49] Quantitative analysis of T-cell clones over time may show diminution of a dominant clone, which may indicate response to therapy.[50]

Differences in HLA alleles and immune responsiveness to HTLV-1 have been identified, suggesting that certain HLA holders may be natural hosts to viral infection because of low immune response. These patients are more at risk for viral-related neoplasia, whereas others, who exhibit greater inflammatory reactions, are more at risk for HTLV-1-related inflammatory disorders.[51] These differences are reflected in populations by immune markers C-reactive protein, soluble CD25, and soluble CD30, which show an activated phenotype in Jamaican patients, but not in Japanese patients.[52]

Molecular Testing for HTLV-1-Related Skin Cancer

IHC for HTLV-1 is not typically used. Anti-Tax IHC has shown limited utility: in 1 study, 6 of 8 ATLL samples showed 1% staining.[53] IHC for gag p19, env gp46, and pX40 Tax has a sensitivity of 45%.

ISH studies of the HTLV-1 mRNA pX, env, and gag indicate technical difficulty.[54] ISH for Tax mRNA lacks sensitivity,[55] possibly because Tax is found in only 40% of ATLL and is decreased in acute ATLL.[42] When positive, Tax-ISH appears to be preserved throughout treatment/response to chemotherapy.[56] Recently, HBZ-ISH was reported using peptide nucleic acid probes to HBZ mRNA sequences, with 100% sensitivity in ATLL.[43] This holds promise, as HBZ mRNA expression is highly conserved in ATLL cells.

SB is most often used at the investigative level to identify clonal patterns of proviral integration and copy number of

integrated viral genomes. Prominent bands suggest HTLV-1 as a driver in an individual neoplasm if they are more oligoclonal than polyclonal, and if the product is found to be integrated near transcription start sites. Tsukasaki et al[57] described 3 patterns of integration based on SB, which significantly correlated with survival. Defective integration, (D type), complete/monoclonal (C type), and multiple/oligoclonal (M type) exhibited a median survival of 6.8, 24.4, and 33.3 months, respectively.

It has been suggested that a high frequency of tumorous clones persisting after chemotherapy ($> 1/300$) may correlate with poor outcome, supporting an aim to achieve the clearance of such clones from peripheral blood.[50] However, the sensitivity and ability to detect clonal change by SB is too low to be used for monitoring of residual disease.[58]

Enzyme-linked immunosorbent assay (ELISA) is widely used in screening and diagnosis of HTLV. Numerous kits are commercially available for immunoassays, each of which uses different combinations of antigenic source, solid phase, and assay type, resulting in a range in sensitivity and specificity.[59,60] The effects of these differences are 3-fold: (1) positive serologic tests must be confirmed by a second specific methodology, either Western blot (WB) or nested PCR; (2) although a negative ELISA test in a low-prevalence community may have a high negative predictive value (NPV), this may be less true in endemic communities[59]; and (3) low-specificity has a high cost to the blood donor pool, where minor numbers of false-positives translate to losses of thousands of donors per year.

WB is a standard confirmatory test for ELISA positivity. WB and/or radioimmunoprecipitation assays identify antibodies to the HTLV viral core (gag, p24) or envelope (env, gp46, gp61/68) proteins. A limitation of WB is the risk of indeterminate results in samples, ranging from 0.02% to 50%.[61] Furthermore, the criteria required for WB positivity varies between public health organizations. Radioimmunoprecipitation assay can further differentiate HTLV-1 from HTLV-2.

RT-PCR is a newer confirmatory test for positive ELISA.[62] Advantages to RT-PCR include the ability to discriminate HTLV-1 and HTLV-2 in the same assay that confirms a positive ELISA and to measure VL. Disadvantages include the cost and inconclusive results, especially when screening large populations. PCR-ISH can clearly resolve the cells that are infected with the virus by using amplified HTLV-1 Tax sequence and probing tissue sections with this sequence.[63]

Population screening programs for HTLV-1 must account for the prevalence of disease in their communities, as well as cost and turn-around time, in choosing their favored screening and confirmation algorithms. Communities with endemic HTLV-1 may need to use multiple techniques before accepting a negative specimen result. In the context of known lymphoma in an HTLV-1-positive patient, additional viral-characterizing studies are not currently used. More correlative assessment of clonal integration patterns, HBZ mRNA expression, and host factors such as HLA types, with outcomes, is needed to develop a rational approach to the use of additional studies.

Herpesviridae (DNA Virus, EBV, KSHV)

EBV has a 90% prevalence in the general population.[64] Skin-localized neoplasms known to be associated with EBV include post-transplant lymphoproliferative diseases (PTLD); NK/T-cell lymphoma, nasal type (NKTCL); nasopharyngeal carcinoma (NPC); and HIV-related EBV+ lymphoma. Other rare primary cutaneous peripheral B- and T-cell lymphomas may be EBV-associated.

EBV is a double-stranded linear DNA virus comprised by the viral genome, nucleocapsid, and viral envelope. The EBV genome encodes approximately 100 viral proteins with regulatory, replicative, immunologic, and structural functions. EBV infects epithelial cells, resulting in active viral replication and cell lysis, and B-lymphocytes, by interacting with the major envelope glycoprotein, gp350, the CD21 molecule,[65] and the major histocompatability complex on the host cell.[66] In resting memory B-cells, infection is followed by circularization of the virus into episomal form and latency. In immunocompetent people, the number of cells latently infected by EBV can remain stable for years.[67]

During latency, a small number of viral proteins are expressed, including 3 membrane proteins, 6 nuclear proteins, and 2 nontranslated RNA types.[64] The nuclear proteins, EBV nuclear antigens 1, 2, and 3 (EBNA 1-3), and EBNA leader protein maintain episomal replication of EBV, inhibit their own proteasomal degradation, upregulate latent membrane proteins (LMP) 1 and 2,[68] and regulate expression of cellular genes. LMP-1 and LMP-2a proteins evade ubiquitination and affect beta-catenin signaling pathways, cell adhesion, and morphogenesis. LMP-1 is thought to play an oncogenic role, by binding to tumor necrosis factor receptor-associated factors in EBV+ lymphomas,[69] resulting in the activation of NF-kappaB, upregulating antiapoptotic proteins, and resulting in B-cell proliferation.[70] LMP-2 prevents EBV reactivation by blocking the activation of protein tyrosine kinases.[71] Nontranslated RNAs (EBV-encoded RNAs [EBERs]) are also thought to be important in tumor development and resistance to apoptosis.[56] Although non-Burkitt EBV-related lymphoproliferative diseases and NPC tend to express LMP-1, LMP-2, and EBER, EBNA-2 and EBNA-3 proteins may be differently expressed.[72]

While most people maintain latent infection with no notable symptoms, infection by EBV is typically followed by rapid development of cellular and humoral responses to the virus, including to the viral capsid antigen (VCA), early antigen (EA), and EBNA. Immunoglobulin G (IgG) to VCA appears in the acute phase, peaks, declines slightly, and persists indefinitely. IgG to EA appears during active infection but becomes undetectable after 3-6 months. Twenty percent of healthy people have this antibody for years. Antibody to EBNA appears 2-4 months after infection and persists indefinitely.[73] The cellular response is mediated by natural killer (NK) cells, CD4+ T cells, and CD8+ T cells, against replicative and latent antigens.[74] Conditions such as immunosuppression and immunosenescence may work against the normal immu-

nologic response, allowing EBV infection to flourish unchecked and predisposing toward cancer.

EBV in Cancer

NPC most often affects the Chinese population in southern China and Southeast Asia, northern Africa, and Alaskan Inuit. NPC is 100% associated with EBV.[64] EBV DNA, measured by EBNA-1 sequences, is found in the blood and in tumor cells from NPC patients. Carcinoma-specific BamH1-A Reading Frame 1 (BARF1) mRNA sequences found in circulation can serve to confirm positive ELISA. It is controversial whether VL correlates with prognosis. Antibodies (IgA) to EBV-EA and VCA are increased in the blood of NPC patients.[75] In contrast to normal controls, antibodies against BRLF-1 protein product Rta have been found only in NPC,[76] and only NPC patients' plasma had abnormal anti-EBV IgG diversity patterns on immunoblots.[77]

Up to 20% of transplant recipients will develop PTLD within 1 year of transplant, 70%-90% of whom are EBV-positive.[78] While EBV-negative patients may have a high risk of developing PTLD on transplantation of an organ from an EBV-positive donor, most PTLDs are thought to result from reactivation of a latent virus in the setting of immunosuppression.[79,80] Risk factors for PTLD include increasing EBV-VL in whole blood and plasma and increased numbers of EBV+ lymphocytes.[81,82] HLA type may further influence risk for PTLD. Decreasing EBV-VL may correlate with response to treatment, and increasing VL may reflect tumor burden.

NKTCL is an aggressive disease with a 5-year mortality rate affecting 50% of the patients, and a predilection for Asian, Central American, and South American populations. Circulating EBV DNA is found in 43%-80% of NKTCL patients' plasma.[83,84] High pretreatment EBV DNA concentrations in plasma may be associated with B-symptoms, high lactate dehydrogenase levels, and high International Prognostic Index scores, and may predict survival after therapy.[83,85] In serial monitoring, EBV DNA VL may correlate with treatment responses and disease stage. Peak EBV level (highest EBV level in the clinical course) appears to correlate with advanced disease stage and may be a more powerful predictor of disease-free survival than the International Prognostic Index. Failure to attain an undetectable EBV DNA in therapy may correlate with a worse overall survival.[83]

Molecular Tests in the Diagnosis and Monitoring of EBV-Associated Diseases

EBV-LMP-1 expression in EBV-associated tumors is variable and limited to subpopulations of tumor cells.[86] The finding of EBV by ISH is the most sensitive/specific means for confirming a role for EBV in tumor tissue, in that ISH localizes virus in tumor cells (Fig. 1B).[87] ISH for EBV uses a probe against EBERs.[88] The test is not quantitative and false-negatives may occur.

In PTLD, serial monitoring of EBV-VL by quantitative PCR may have utility in early detection of disease, and is often performed for this reason and for monitoring for treatment response, but the positive predictive value (PPV) is low. In

NKTCL, EBV DNA monitoring is sometimes used to determine treatment algorithms and provide prognostic information. It has been noted in NPC studies that circulating EBV-DNA plasma values measured by qRT-PCR depend on the size of the amplicon measured and do not correlate with anti-EBV IgG or IgA ELISA. Even so, plasma EBV DNA VL is recommended by some.[89,90] Suggested confirmation tests to the EBNA-1/VCAp18 screen include ELISA for IgA EA[91] or anti-EBV IGG diversity patterns on immunoblot,[77] which may also predict relapse.[92] In the Southeast Asian population, noninvasive tests such as nasopharyngeal brushing are used in screening for NPC. Diagnostic cutoffs for PCR-quantified VL from these tests, equating with presence of the malignancy, have a sensitivity of 98%, specificity of 90%, PPV of 97%, and NPV of 91%.[89]

Human Herpes Virus 8

KSHV, or HHV-8, a double-stranded DNA virus, causes a long-term viral infection, latently infecting endothelial cells and lymphocytes, in episomal circularized form, preceding cell immortalization and transformation.[93] While in the lytic phase, HHV-8 expresses a number of immediate early, early, and late transcripts; in latency, HHV-8 encodes KSHV-specific antigens, including K12, K13/viral FADD-like interferon converting enzyme inhibitory protein (vFLIP), vCyclin, and latency associated nuclear antigen (LANA)-1.[94] While latent proteins play a role in the survival and proliferation of tumor cells, lytic proteins are thought to contribute to tumorigenic microenvironment alterations.[94] LANA-1, homologous to EBV EBNA-1 protein, may cause dysfunction of cell-cycle regulatory checkpoints by degrading p53 and inactivating pRb.[93] HHV-8 antigens target cell signaling pathways, and deregulate apoptosis and immune response[93] through vCyclin, vFLIP, bcl-2 oncogene, viral interferon regulating factor, and vIL-6.

Phylogenetic analysis has elucidated at least 8 HHV-8 subtypes (A/C, J, K/M, D/E, B, Q, R, and N), which have geographic associations.[95] HHV-8 has a prevalence of 3%-25% among U.S. and European donors. In Uganda, Gambia, and the Ivory Coast, seropositivity ranges from 50% to 100%. Endemic areas are also known in South America. HHV-8 is thought to be cotransmitted with HIV-2, especially through an anogenital route. HIV coinfection amplifies the incidence of Kaposi sarcoma (KS) by 70,000 times. Organ transplant recipients are also at risk for KS.[96]

HHV-8 in Cancer

IHC for HHV-8 LANA protein has a 99% sensitivity and 100% specificity for KS,[97] and is a useful diagnostic adjunct (Fig. 1C). HHV-8 RNA can be detected in situ for many transcripts, including LANA, vCyclin, vFLIP, KaposinB, Rta, and vIRF-1.[94] HHV-8 DNA is found in peripheral blood of patients with KS,[98] as well as in all lesions of KS, regardless of HIV status. HHV-8+ patients with KS have higher viral titers in peripheral blood than HHV-8+ patients without KS. Although the development of antibodies to HHV-8 can predict

the development of KS in HIV/AIDS, the value of this test is questionable, as the clinical and pathologic presentation of KS is characteristic. Lytic antibodies and VL in blood, serum, and saliva increase in higher stages (III/IV). RT-PCR showed that HHV-8 strain A is found in rapid progressors and is associated with higher VL, and type C is found in slower progressors.[99] Type E HHV-8, endemic in Amerindian tribes, does not seem to highly increase the risk for KS, of which there is a low incidence in this population.[100]

Polyomaviridae (DNA Virus, Merkel Cell Polyomavirus)

Merkel cell polyomavirus (MCV) is a small double-stranded DNA virus, composed of a circular genome encoding a large T antigen (LTA), small T antigen, micro-RNA, and viral capsid proteins VP1, VP2, and VP3.[101,102] MCV may integrate into the host genome or be present in episomal form. MCV is known to exist in at least 4 strains: TKS, MCC339, MKL-1, and MCC350; strains in Europe appear conserved and are similar to the MCC339 strain in the United States and MKL-1 in Sweden.[101]

MCV is present in up to 80% of healthy blood donors and 94% of normal skin samples from healthy patients, as well as in nasal swabs and urine.[103] Infection, which typically occurs in childhood,[104] is asymptomatic before, during, and after seroconversion.[105] After infection, serum levels of immunoglobulin M may peak before, with, or 1-2 years after seroconversion to IgG.[106] Anti-MCV antibodies are found in 40%-88% of adults.[101,107]

MCV in Cancer

Merkel cell carcinoma (MCC) is an aggressive primary cutaneous neuroendocrine tumor, often associated with immunosuppression. MCV is present in 43%-100% of MCC specimens.[108-110] IHC with monoclonal antibody 2B4 against the LTA of MCV shows slightly fewer MCC to be positive. Clonal integration is an early event in MCC development. In integrated MCV, truncating mutations of the LTA suppress the helicase domain and abort viral replication.[111] LTA appears to bind the tumor suppressor Rb. Silencing LTA in MCC cell lines generally stops the tumor phenotype, suggesting that LTA is required for maintenance of most MCC.[112] MCV small T antigen also acts in an oncogenic manner, dysregulating cap-dependent translation.[113] Up to 29.9% of non-MCC cancers tested have been found to contain some evidence of MCV, although none so uniformly and consistently as MCC, suggesting a lack of specificity of PCR as a diagnostic adjunct for MCC.[114] High VLs are only typically seen in MCC.

Molecular Tests in the Diagnosis of MCC

Nested PCR can be used to identify MCV sequences in tissue. Several primer pairs are available for different sequences; LT3 has been said to have greater reliability compared with LT1 or VP1.[115] MCV DNA is present or absent similarly in MCC tissue versus perilesional skin, but at a higher level in tumor tissue than in perilesional skin. MCV DNA has been found in 10% of benign lymph nodes tested, supporting the role of the lymphocytes as a reservoir.[116] MCV DNA VL is higher in patients with MCC than in those without MCC, as are titers.[107] The presence of a lower VL (< 1 copy per cell) may correlate with worse outcome, including a poorer response to therapy and distant metastasis, whereas a higher VL may correlate with longer survival,[103] although controversial.[117] Immunosuppression correlates with much higher VL.

IgG to VP1 is found in all patients with MCC, and 85% of controls, and is detectable up to 25 years after exposure. Anticapsid IgG is higher in patients with MCC than without. It has been suggested that monitoring MCV antibodies may be helpful in monitoring tumor progression. In patients with tumors; antibody titers > 10,000 may signify a better progression free survival, and lower titers may suggest early recurrence.[101] Post-treatment, however, anti-VP1 antibodies fell in patients who did not recur and rose, predicting metastasis, in those who progressed.[118] There is a suggestion to classify MCC patients into 2 groups: (1) those with high VL/LTA+/high anti-MCV antibodies, and retinoblastoma expression, who have better survival; or (2) low VL/LTA−/low antibodies.[101,119] Compared with VP1 antibodies, MCV T-Ag/LTA IgG is more specific to MCC, correlates strongly with MCV VL and LTA expression in tumor cells, and appears to correlate with disease course; LTA antibodies decrease with successful treatment of MCC, and rising titers appear to predict progression.[118]

IHC with monoclonal antibody 2B4 (CM2B4, Fig. 1D) may be useful in the diagnosis of neuroendocrine tumors of unknown primary. VL and VP1 or LTA serology are controversial but may ultimately be useful in prognostication of disease.

Conclusions

Tumor viruses are playing a growing role in understanding cancer pathogenesis. These viruses may affect key cellular machinery or change microenvironments and have a relationship with host factors. Better knowledge of the action of these viruses on host cells and the resultant cellular effects is pivotal in development of anticancer strategies. In the future, collaborative efforts will help to validate assays and define algorithms for tumor viruses' tests.

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