Molecular Signatures of Infectious Agents

Summary and Recommendations

Molecular Signatures of Virally-Induced Cancers: a Rationale

Viruses are associated with at least 15% of human cancer which is likely to be an underestimation of the actual contribution of viruses to human cancers. Research should be encouraged that will help to identify individuals exposed to infectious agents and who are subsequently placed at increased risk of developing cancer. Since in many cases the infectious agents are ubiquitous, the key is in identifying that subpopulation of exposed individuals who are likely to develop cancer as a consequence of the initial exposure to the infectious agent. Identifying this subpopulation represents the needle in the haystack dilemma; however, molecular markers that help to identify the subpopulation at risk of cancer provide a potential solution. Necessarily, such molecular markers must distinguish between infections per se and those infections that are contributing to development of cancer. As such, it is not unreasonable to predict that to make such distinctions will require identifying differing patterns of expression of multiple markers, i.e. generating a molecular signature for a particular cancer. These molecular signatures must permit the reliable and accurate identification of individuals-at-risk at an earlier enough stage in cancer development such that intervention can be effective. The information gained from defining molecular signatures for cancers should provide potential diagnostic tools in addition to potential insight into the mechanisms of action of viruses in cancer. These studies may also define potential targets for new modalities of cancer therapy, though this goal must be considered too broad to address in the context of this proposed programmatic effort.

As indicated above, a likely deficit in our knowledge of cancer etiology is in identifying the role of existing and novel viruses in cancers which today are not recognized to be virally caused. There are numerous examples today of human cancers for which a viral etiology has been proposed but not established. For instance, the controversial role of SV40 and human polyomaviruses in CNS (central nervous system) tumors, the recently raised potential role of EBV (Epstein-Barr virus) in breast cancer, and the argued role of TTV in liver cancer need further investigation. It is reasonable to speculate that other human cancers are caused by viruses, and that some of these viruses may not yet be identified. Molecular signatures for virally-associated cancers, as part of a comprehensive program encompassing epidemiological research, basic virology and cancer biology, may help us identify existing and/or novel viruses that contribute to additional human cancers. For example, if it were to be discovered that human papillomaviruses (HPV) cause specific changes in cervical cancer cells as they progress towards cancer, and were these changes found to reflect the function of virally encoded gene products, then it would be reasonable to look for whether similar changes arise in cancers at other anatomical sites in which HPV is suspected but has not been proven to be a causal agent (e.g. certain skin cancers, oral cancers). Many oncogenic viruses appear to target a similar set of cellular targets, leading to their inactivation and/or deregulation. For instance, HPVs, SV40, Py, and adenoviruses all target the Rb and p53 tumor suppressor genes, as well as p300/CBP. The combined effect of these disruptions is likely to result in certain alterations in the pattern of gene expression and function. Furthermore, these alterations arise in the absence of mutational events that otherwise occur in cancers in which viruses are suspected to be contributory. These differences potentially could be exploited to identify cancers caused by unknown or unappreciated oncogenic viruses which act through a similar mechanism. This very approach is being used currently to assess the causal relationship of SV40 in human tumors. Viruses tend to modulate innate immune responses of the infected cell; evidence of these changes in a cancerous or precancerous lesion may provide a flag in identifying cancers in which a viral etiology exists. Thus, a programmatic focus on developing molecular signatures for virally-induced cancers may provide not only valuable diagnostic tools for early detection of cancers with known viral etiology, but also help define roles for viruses in human cancers without a known viral etiology.

Workshop Summary

Dr. Peter Greenwald, Director of the Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, welcomed workshop speakers and participants and emphasized that molecular signatures of infectious agents have important potential for cancer prevention. Dr. Greenwald said that his expectation is that recommendations from the workshop will suggest areas of future research for the NCI, especially in the field of cancer screening and detection and the identification of biomarkers of disease.

Dr. Sudhir Srivastava, Chief of the Cancer Biomarkers Research Group, Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, presented background information on the biological paradigm of cancer detection for secondary prevention and intervention. He presented data on the prevalence of cancer at sites commonly affected by infectious agents. Dr. Srivastava explained how extramural research is supported by the Cancer Biomarkers Research Group and the Division of Cancer Prevention to improve the understanding of molecular signatures of infectious disease. Dr. Srivastava provided an overview of the Early Detection Research Network (EDRN), a DCP initiative to identify cancer biomarkers and to explore the use of infectious agents as possible biomarkers for specific cancers. The EDRN, established in 1999, involves more than 200 investigators at 30 institutions and is supported by 18 Biomarkers Developmental Laboratories, two Biomarkers Validation Laboratories, one Data Management and Coordinating Center and eight Clinical and Epidemiological Centers. The EDRN Steering Committee and Advisory Committee provide EDRN oversight and guidance for setting research agendas. Because viruses may play an important role in cancer etiology, it is important that EDRN consider their use as biomarkers of disease.

Dr. Mukesh Verma, Program Director in the Cancer Biomarkers Research Group, Division of Cancer Prevention, National Cancer Institute, National Institutes of Health, presented the following objectives of the workshop:

- Review state-of-the-science in detection technology that can identify extraneous genomic insertion in human cancers; and
- Recommend future research directions for using the molecular signatures of infectious agents for early detection, risk assessment, and prevention of cancer.

Dr. Verma identified issues that served to focus of the workshop presentations and identified ideal outcomes sought from the workshop. Each of the two sessions will focus on distinct areas of research: Session I will focus on advances in technology that impact the early detection of infectious agents in human cancer, and Session II will focus on the contribution of infectious agents in the development of cancer and in prevention strategies. Recommendations from the workshop will be made based on what research priorities are identified as key to advancing the field of cancer prevention and treatment.

Dr. Paul Lambert, Professor of Oncology at University of Wisconsin Medical School, presented the keynote address for the workshop. Dr. Lambert emphasized the need to develop methods to identify individuals at risk for developing virally-induced cancers, with the ultimate outcome to develop intervention strategies. The use of epidemiology to establish correlations between infection and increased incidence of cancer, the development of appropriate experimental models, and the identification of molecular signatures as markers of disease are important issues to be investigated. Dr. Lambert presented information on viruses that appear to be associated with cancer, such as human papilloma virus (HPV) in cervical cancer. The contribution to carcinogenetic progression was described for an animal model using transgenic mice. He described the role of genetics in determinating progression to cancer and the interactions between various molecular targets of the HPV virus. Results from ongoing research indicate there are multiple mechanisms for tumor initiation and promotion. This is the case in HPV-16, in which two viral genes, E6 and E7, are commonly expressed in human cervical cancer cells. In a study in transgenic mice, E6 acts sparingly at the promotion stage but strongly at the progression stage of

tumor growth. The E7 gene acts in the opposite manner. These findings highlight the importance of identifying genes of infectious agents in development of cancer to determine their specific role at each stage of cancer development.

Dr. Lambert stated that determining causal relationships between infectious agents and cancer is only one priority for future research. Other priorities include determining what viruses cause specific cancers, the role of infectious agents in mortality, the likelihood of interventions in prevention of disease, the identification of known and unknown viruses with regard to cancer, and the development of a proof-of-principle to characterize the virus/cancer paradigm.

Dr. Timothy M. Block, Director and Professor at The Jefferson Center, Thomas Jefferson University, discussed the use of two-dimensional gel electrophoresis (2-DE) as a tool for the systematic analysis of polypeptides. 2-DE is being used to identify serum polypeptides associated with the onset and progression of Hepatitis B (HBV) and Hepatitis C (HCV). This proteomic approach has the possibility of being applied to other diseases.

Dr. Block discussed the incidence, mortality, and pathology of HBV and HCV and their role in liver cancer. Because hepatocellular carcinoma (HCC) has a long latency before disease is apparent, there is an unusually long period when prevention of HCC is possible. He explained the state-of-the-science in the field of therapeutics for the hepatitis viruses, such as glucosidase inhibitors, which seem to be virus-specific and have a minimal cytotoxic effect on the host cell.

Proteomics research focuses on the identification of individuals infected with HBV and HCV who are likely to develop HCC. Dr. Block presented progress made in developing techniques and software that can identify biomarkers and stressed that these investigations must continue in order to refine the technique and confirm their importance in molecular signatures of infectious disease. The research on alterations in oligosaccharides in hepatitis infection has led to the development of "glycomics," which has promise as a new class of diagnostic markers. Dr. Block has used total serum from patients for proteomics analysis and has purified Hepatitis B-subviral particles for glycomics.

A question arose regarding the type of serum used in this research, and Dr. Block explained that for proteomics, his lab uses a patient's total protein serum; for glycomics, patient's total protein serum is used but with purified Hepatitis B-subviral particles. A participant asked if glycoform variability presents a problem in proteomics. Dr. Block replied that glycoform variability has not been a problem, but they may have to consider other analyses in the future. He added that a limitation of 2-DE is detecting small quantities of proteins or protein products.

Dr. Block also responded to a question about whether HCC may be affected by a co-carcinogen. His reply indicated that a true proteome with enough data collected will allow one to distinguish different etiologies and provide clues to pattern clusters that are typical of a cancer caused by different agents.

Dr. Kamal Khalili, Director of the Center for Neurovirology and Cancer Biology, Temple University, presented background material on JC virus (JCV). JCV has been associated with oncogenic properties through its T-antigen, which suppresses the genes p53 and pRb. JCV is responsible for dysmyelination diseases of the central nervous system in immunosuppressed individuals. Dr. Khalili explained the possible role of JCV in human tumors, including medulloblastomas, pilocytic astrocytomas, and oligoastrocytomas. Evidence of JCV T-antigen was evident in each tumor type, making JCV or its proteins potential molecular markers for human cancer. Dr. Khalili presented results demonstrating *b*-catenin expression in mouse and human medulloblastoma and suggested that *b*-catenin also may be a possible molecular marker for the development of tumors associated with JCV.

A participant asked why some cells showed scattered T-antigen staining. Dr. Khalili replied that only about 20-30 percent of the cells are positive for Tag, but 100% are positive in transgenic mice. On whether these cells were SV40 positive, Dr. Khalili said it was not present in serum, but one cell was positive by polymerase chain reaction (PCR). As to the possible promoter of JCV, Dr. Khalili said JCV acts as its own promoter. To other questions, he said his research group has not looked at JCV in kidney cells. His lab has used many antibodies in the research, including

SV40 and JCV T-antigen, but they have not investigated JCV in other human dysmyelination diseases.

Dr. Elizabeth R. Unger, Acting Chief of the Human Papillomavirus Section, Centers for Disease Control and Prevention (CDC), provided background data on the role of HPV in cervical cancer. More than 12,800 new cases and 4,800 deaths occur from cervical cancer. The cost of followup tests for abnormal PAP smears is $8B a year. Dr. Unger described the association of HPV to cervical cancer and the need to identify specific types of HPV, of the 70 types currently identified, which may be more important than others in the development of cervical cancer. There are two phylogenetic branches of HPV-cutaneous and mucosal, and each has its own role in cervical cancer or other disease states. HPV is a good candidate for investigation as a biomarker for cervical cancer, although it is not known which infected cells will progress to cancer or which variants of HPV may be more likely to cause cancer. The use of a molecular PAP smear is on the horizon and should be an important addition to the screening regimen for cervical cancer.

Dr. Magnus von Knebel Doeberitz, Professor of Molecular Oncology, at University of Heidelberg, Germany, began his presentation by describing that the biggest challenge in identifying molecular signatures is gaining the ability to select biomarkers that can determine which lesions will progress to cancer from those that will not. This objective should be the purpose of developing new tools for cancer screening. Dr. von Knebel Doeberitz described mechanisms in HPV that may be used to classify certain HPVs as "high risk" and the different oncogenes (e.g., E6 and E7) that they express.

Among the new molecular tools discussed by Dr. von Knebel Doeberitz was a PCR assay for the amplification of papillomavirus oncogene transcripts (APOT). APOT may have an application in identifying a specific progression marker in future cervical cancer screening protocols.

A participant asked if the APOT test could be carried out on normal cervical mucosa. Dr. von Knebel Doeberitz replied that it was possible using a cytobrush or some of the new media available for cytology. To a question on classification of cervical intra-epithelial neoplasia (CIN) lesions, Dr. von Knebel Doeberitz replied that he differentiated classes of CIN and found the prevalence of integrated HPV genome was approximately 40 percent. Another participant asked if he had followed up on patients classified as 'normal' or 'low risk' at the beginning of the study to see if they changed classification in subsequent tests. Dr. von Knebel Doeberitz replied that all samples that tested positive for the p16 gene (high risk HPV) were followed up.

Dr. Susan Marriott, Associate Professor in the Division of Molecular Virology and Microbiology, Baylor School of Medicine, presented information on human T-cell leukemia virus type 1 (HTLV-1) Tax protein, a transcriptional transactivator and viral oncogene. HTLV-1 is associated with adult T-cell leukemia and tropical spastic paraparesis, each characterized by infection of CD4+ T-cells. Each of these diseases occurs in only approximately five percent of infected individuals and can have a latency of 20-40 years. A Tax-reactivated gene, proliferating cell nuclear antigen (PCNA), is important in DNA replication and repair and is overly expressed in almost all tumors and transformed cell lines. Dr. Marriott described research showing that Tax suppresses DNA repair while stimulating DNA replication, thus contributing to the monoclonal nature of HTLV-1 transformed cells.

Dr. Jae Jung, Chairman of the Tumor Virology Division, New England Regional Primate Center, Harvard Medical School, described the mechanisms used by viruses to target and modulate various aspects of the host's immune system. Dr. Jung's research focused on human herpesvirus-8 (HHV-8) and Kaposi's sarcoma-associated herpesvirus (KSHV). He described the synergistic effects of B7-2 and ICAM-1, ligands of the natural killer (NK) cell-mediated cytotoxicity receptors, and the manner in which they are downregulated by KSHV. This may be the primary mechanism for infection. K3 and K5 proteins are zinc-finger membrane proteins encoded by KSHV and are significant inhibitors of immune response; for example, K5 suppresses the B7-2 and ICAM-1 surface expressions and allows the KSHV to avoid NK immunity.

A participant asked if Dr. Jung had the opportunity to study HHV-8 isolates from different parts of the world to see if the pirated genes are similar among different isolates and if there is any correlation with disease potential. Dr. Jung responded that he has looked at various isolates and

there is no apparent sequence or functional difference. Dr. Jung responded to another question that all viruses studied were expressed in the lytic rather than the latent phase. There is no viral expression during the latent phase.

Dr. Betty L. Slagle, Assistant Professor of the Department of Molecular Virology and Microbiology, Baylor College of Medicine, presented information on the association of HBV and environmental carcinogens as primary risk factors for HCC. In transgenic mice, it has been shown that a cofactor, HBx transactivating protein, is associated with inhibition of DNA repair functions of human and murine cells damaged by either ultraviolet light or aflatoxin B1 exposure. One hypothesis is that HBx inhibits DNA repair in chronic HBV infection. The ATX mouse model has been used to show that HBx does inhibit DNA repair. Dr. Slagle described experiments in transgenic mice that show the potential of this model in liver cancer and the possibility of better defining the role of HBx as a cofactor that is a tumor promoter.

A participant asked if Dr. Slagle investigated bromodeoxyuridine (BrdU) incorporation in liver cells to see if PCNA is pushing the cells through the cell cycle. Dr. Slagle responded that BrdU incorporation was used to determine if this was the case, and it was confirmed. She also clarified that HBx expression is confined to the liver.

Dr. Robert L. Garcea, Professor of Pediatrics of the University of Colorado School of Medicine, presented an overview of simian virus 40 (SV40) and discussed the possibility that it may be a human pathogen. SV40 genomic sequences have been found in various human tumor samples, including choroid plexus neoplasms, ependymomas, osteosarcomas, and mesotheliomas, and is virtually identical to that of the human polyomaviruses BK and JC. Dr. Garcea described regions of the SV40 genome that are similar to other human viruses and reviewed the theory that the virus may be associated with infection through human polio vaccines in the 1950s and 1960s. He described the findings from PCR investigations as a base for the evolving theory that SV40 may be a human pathogen. Dr. Garcea recognized that this area is controversial but may be valuable to investigate because recent research results and advanced laboratory techniques make it possible to answer this question. He listed research results in support of SV40 as a human pathogen and results that dispute the premise.

One participant commented that although PCR may not be adequate to completely answer these questions, it is useful for the identification of SV40 in various cell and tumor types. Another participant commented that a recent article reported that familial Li-Fraumeni syndrome harbors a deletion in the p53 gene, exon 4, that overlaps with JCV T-antigen in negative transgenic mice. A participant reported that research in the 1960s showed that prisoners who were either given the polio vaccine contaminated with SV40 or possibly given SV40 directly were later found to have replicated SV40 in feces samples. Dr. Garcea added that this type of experiment was reported from various locations. Dr. Garcea also noted that it would be difficult to investigate SV40 using a "hit and run" strategy.

Dr. Laimonis A. Laimins, Professor in the Department of Microbiology-Immunology, Northwestern University Medical School, discussed changes in cellular expression induced by HPV and their association with high risk genotypes (i.e., HPV-31) that include tumors and low risk genotypes (HPV-11) that induce benign lesions. Microarray analysis allows investigation of transfected keratinocytes and identification of genes activated by HPV-31 gene products. Results indicate that of approximately 7,200 genes investigated, about 170 genes were activated, although no particular pattern emerged to account for the affect of the virus. However, genes repressed by HPV-31 included distinct families of genes, such as the interferon inducible genes, which may play a role in immune susceptibility by reducing the protein Signal Transducer and Activator of Transcription (STAT-1). Dr. Laimins compared the expression of genes by HPV-31 and those expressed by HPV-11.

A participant asked if Dr. Laimins could identify certain proteins that HPV inactivates in the interferon pathways. Dr. Laimins responded that one hypothesis suggests that HPV gene products act to repress expression of STAT-1, which impairs the entire interferon response pathway. He added that in addition to E6, E7, and E2, other components of the interferon pathway may be involved. Another participant asked if there is normal induced interferon in viral

infections. Dr. Laimins responded this is difficult to show in experimental models because the infected cells begin with a repressed level. For a question on the possibility of knocking out STAT-1 with antisense, Dr. Laimins answered that this may be a valuable step to determine if the viral infection could be reproduced.

Dr. Lambert introduced the discussion topics for both sessions and explained that participants should consider the information presented earlier to develop recommendations for each of the issues. Of particular importance is identifying areas that may be key to improving early detection of cancer and identifying gaps in knowledge that preclude use of molecular signatures of infectious disease for cancer prevention and intervention strategies. Also, increasing our ability to screen patients would identify those at increased risk for developing cancer and would be important for reducing the societal burden of cancer. Dr. Lambert asked that each participant consider the discussion topics in terms of particular viruses of interest to research in their own field.

The first topic for discussion pertained to the significance of understanding the molecular signatures of infectious agents in human cancer. Preliminary to the first topic is the definition of molecular signature and which new markers need to be developed. Infectious agents are a valid field of study for cancer researchers. A perplexing problem is the number of viral variants that exist and the possibility that a variant may be associated with the disease but the normal virus (non-variant) may not. Epstein-Barr virus (EBV) is a good example because it is associated with more than one malignancy and there are multiple variants of EBV. Understanding the role of variants should become a research priority. In fact, there is evidence that some EBVs may be geographically related to certain malignancies, which complicates this even more. Herpes viruses display numerous variants, but there is no conclusive evidence that suggests one variant is more important than others in contributing to disease. The human polyomavirus JCV also has variants, but there is no evidence that suggests that there are more virulent strains than others.

The characterization of molecular signatures of viruses is important from a diagnostic standpoint and may prove beneficial in the development of new therapeutic agents for prevention or treatment of cancer. One problem may be the term "infectious," which has associations for researchers, physicians, and patients that do not accurately characterize their role in cancer etiology. In cancers, the implication of infection surpasses their action as infectious agents; they are acting, through integration as in the case of HPV, as an extra piece of DNA. It also is important to investigate the role of infectious agents in the development of early lesions that progress to cancer. There is very little understanding of the etiology associated with lesions that progress to cancer compared to those that regress. In general, there is a need to study the stages of cancer by a better understanding of the natural history of viruses. Intervening at early viral stages of the disease may allow more successful prevention or treatment.

Of critical importance is finding the imprint that the virus leaves on the host cell and then using that information as an opportunity for intervention. Molecular signatures will more clearly define etiology, which should lead to prevention and therapies that are based on earlier diagnoses. HBV is an example where we know that the virus plays a major role in the etiology of liver cancer; thus if you prevent HBV infection, you reduce the incidence of liver cancer. Whether or not you treat the cancer or treat the target of the cancer, molecular signatures should suggest an intervention.

Another issue in early detection is defining the means of treatment for identified infections if investigators suspect the infection will lead to disease. Again, HPV can be used to illustrate this problem. HPV is not easily recognized by the immune system, and the PAP smear is currently not able to identify cytologic changes specifically caused by variants of HPV. If we can identify molecular signatures of a stage of cancer too late for successful intervention strategies, it may be better to develop molecular signatures of earlier-stage disease.

Focusing on novel agents, like HHV-8, is a daunting task, but program would have an enormous impact in terms of improving the public health. Meanwhile, there is a need to continue to screen tumors for the presence of viruses while investigating the role of known viruses in the etiology of cancer.

There may be a benefit from investigating known and discovering unknown viruses. One strategy might be to synchronize this initiative collaborate with existing initiatives, such as the biomarkers research in the EDRN, to get support in searching for infectious agents. The initiative to find molecular signatures of infectious disease might benefit from collaboration with the EDRN, both for intellectual collaboration and sharing of tissue samples. This approach could be used for network-sharing, reagent-swapping, and technology-sharing.

A discussion ensued on the availability of funding, either from the NCI or other institutions, for research that is considered high-risk/high-impact. Dr. Srivastava explained the types of funding available through the NCI to encourage such research. He presented information on R21 and R33 NCI grants that support exploratory research considered high-risk/high-impact. After a discussion of funding mechanisms and how this type of research could be structured, participants were encouraged to submit applications in two areas: research on the means of finding infectious agents related to cancer, and the use of technologies or methodologies for identifying agents. As long as the justification for the research is sound, applications will be considered.

Burkitt's lymphoma (where EBV infection is involved) is not recognized by cell-mediated immune mechanisms because an antigen suppresses the normal human leucocyte antigen (HLA) response. However, in EBV lymphoproliferative syndromes, which are virus-driven diseases arising rapidly in immune deficient patients, specific cytotoxic T-cells can be used for therapy, unlike in Burkitt's lymphoma. In liver cancer, folate deficiency also can affect the progression of the disease and other nutritional factors may infringe on the immune status of patients. Methylation of DNA might be a contributor to risk in many of these diseases.

In cervical cancer, haplotypes differ and confer different risk or protection; however, the literature on this is very inconsistent. Haplotypes in class-II genes and extended haplotypes that include class-I-linked genes have associations with risk and protection in cervical cancer, presumably related to immune function. Research needs to be directed toward cellular-immune responses in HLA-defined individuals. Genome-scanning using overlapping peptides and examining cytokine-expression as it relates to class-I and class-II responses is needed to tell how host-immune responses dictate the outcome of the viral infection. HCV may be a virus that acts in this manner.

The role of immunity was discussed in other host-virus systems also. In experimental studies, the link between JCV and immune response has not been confirmed. In human immunodeficiency virus (HIV), there has been no increase in cancer risk among those infected, which indicates that the role of immunity in cancer must be questioned in light of these findings. This is true in HIV patients for HPV, but this is related to high-risk behaviors that possibly preceded the HIV infection. In summary, the broader theme needs to be defining the role of the immune system in viral oncology. In some cases, such as HBV and HCV, there is a clear immunopathology present in activating the immune system. As related to molecular signatures, or the impact that a virus has on the host, immunopatholgy should be considered as a valid area of inquiry.

On the second day of the workshop, Dr. Ronald Desrosiers of the Harvard Medical School provided background on HIV, a member of the lentivirus subfamily of retroviruses, many of which are oncogenic. Lentiviruses are not considered oncogenic but may cause long-term chronic disease. There is no evidence that lentivirus has a role in cell transformation, although one investigator reported monoclonal integration of HIV-1 sequences in macrophages of mixed-cell lymphomas. Cancers that arise through HIV infection are basically opportunistic neoplasms, in most cases virally-associated. The most common cancers associated with HIV-1 are B-cell lymphomas (predominantly EBV positive), Kaposi's Sarcoma (KS), and cervical carcinoma with HPV associations.

A discussion ensued of HIV infection and its effect on the immune system. There is a hypothesis that in the early weeks of infection, the viral-specific CD4+ T-helper cells congregate at the site of HIV infection, generally lymphoid tissue, and the HIV proceeds to eliminate them. When HIV eliminates the CD4+ T cells, a primary defensive strategy of the immune system, HIV is allowed to replicate unrestrained for months.

Molecular signatures are needed in EBV-associated lymphomas because lymphomas arise in tissue (e.g., brain) where they are very hard to diagnose. Lymphomas are polyclonal but end up

monoclonal and therapy does not work until the advent of EBV-specific T-cell cytotoxic agents, suggesting that EBV plays a major role in causing lymphoma. Molecular signatures could be used for two approaches. One use of molecular signatures could elucidate if there is cell proliferation in latently-infected cells. Another use is to exploit lymphoma-expressed EBV latency antigens, therefore, it may theoretically be able to identify these cells as they are released into the general circulation.

A number of biological proteins may also be valuable biomarkers, such as immunoglobulins. Nasopharyngeal carcinoma (NPC) is a condition where there is a circulating immunoglobulin (Ig) A response to EBV replicating antigens. IgA could be used as a molecular signature for early onset of NPC, especially in high-risk groups. The EBV is latent until late in life, and co-factors, such as genetic factors or nutritional co-factors, make it possible for EBV to infect epithelial cells on transporter pieces of IgA. The NPC can then take hold. There is no indication that IgA would be a useful marker for HPV, but there are antibodies to HPV genes E6 and E7 in cervical cancer. In addition, systemic IgG is measurable and correlated with exposure to HPV and does represent level the of infection.

Dr. Desrosiers also introduced the topic of animal models in understanding the host-virus interaction by explaining the usefulness of animal models and different methodologies being used in animal models. The direct method-infecting an animal with a human virus-is the ideal animal model, but there are very few of these viruses that are able to replicate in animals (EBV in new world primates). Instead of the direct method, most of the current animal models use the analog method where an animal virus that is similar to a human virus is used (Rhesus EBV, a Rhesus cryptovirus) because it is genetically similar.

Transgenic animal models are becoming more common and may become more valuable for evaluating viral infectivity in cancer in the future. There are HPV model systems in mice that are limited in success. A monkey model for HPV-16 is in early development and looks promising. The use of hamsters, rats, and new world monkeys in models of JCV and brain tumors has been developed over the past two decades and is very beneficial in understanding astrocytomas. However, more research is needed to make the model more applicable to human studies. The same is true of the woodchuck model for Hepatitis B where transgenic models also are being used. While there are no good animal models for Hepatitis C yet, one is under development in new world monkeys.

Participants discussed the reasons why there is not an HBV-like virus in monkeys that is equivalent to the human virus. Because HBV equivalents are found in woodchucks and ducks have endemic infection of HBV, it seems logical that it should be found in monkeys. Panthers and turtles also harbor a virus homolog of HBV. This led to a suggestion that it would be beneficial to develop a taxonomy of viruses associated with cancer to see if oncogenes are the same in humans and in animal models. This classification has been done for a few viruses, such as HHV and HPV, and there are subgroups that are strongly associated with cancer and some that are only weakly associated. There are many confounding variables that also might be important, including lifespan, environmental factors, host-susceptibility factors, and diet.

Dr. Desrosiers introduced the topic of SV40 in humans. He reported that the data for SV40 sequences in specific tumors is compelling and more attention may need to be focused on this. Research is needed to characterize naturally occurring SV40 isolates. Some examples of SV40 infection in tumors, such as mesothelioma, were presented, and theories of the origin of SV40 in animals used in polio research were discussed. It was determined that this may be an opportunity to investigate some of the questions discussed by participants in this workshop. There also is a need to prospectively collect samples from subjects who are likely to develop the disease later in life. Molecular signatures can help identify those individuals.

The role of host immune response in viral infection was also discussed. This is an area that also can benefit from collection of prospective samples before there is an identified molecular signature involving the immune system. One problem is that there is not a clear understanding of which specific immune marker(s) may be used to identify cancer. Most of the immune system components are activated by any inflammatory condition, not just cancer. It may take specific

tests, such as antigen stimulation, to separate markers caused by cancer. A current example of a valid immune marker in cancer is IgA in NPC.

Another interesting topic " molecular piracy" reported first in KHSV was also discussed. Molecular piracy refers to viruses that capture host genes, such as insertion in Bcl-2, which may or may not be essential for tumor development. For instance, EBV must stimulate the production of interleukin (IL)-6 and IL-8 in order to replicate, and these cytokines may be useful as valid molecular signatures. Most of the captured genes are not required for viral replication or pathogenesis. Some are involved in viral reactivation or deactivation and may be useful as molecular signatures.

There is a need for creative approaches to develop new therapeutics or prophylactics for cancer prevention and treatment. In HPV, there is an initiative to create immunological reagent vaccines that contain the E6 and E7 oncoproteins of HPV-16 in hopes that those oncoproteins may be present in cancer. For the HBV vaccine, there is the use of the virus in different forms, as well as different gene products.

An issue that needs clarification is the ability to use molecular signatures to identify early enough in the disease process to stop the cancer process. Retinoids are being considered for NPC to prevent secondary tumors. An antisense oligo deoxyribonucleotide (ODN) approach to cure EBV latent infection has been tried, but it was very difficult to stop growth of these cells. The antisense approach in humans would be very difficult because of the problem with administration, although there has been some apparent success in human leukemia. The biodistribution of antisense compounds also makes them very difficult to use in animal or human studies.

The discussion on the second day also included intervention and therapeutic approaches to viral infection. There is research underway to create an EBV vaccine, but because EBV infects epithelial tissues, there has not been much progress to date. When infection occurs in B-cells, a vaccine may not be possible. This is an area that should be of interest to the research community. HBV vaccine trials are ongoing and show promise, but this approach is very expensive. There is an urgent need for less expensive vaccines.

Recommendations

Below are listed the several interrelated areas in need of support in developing molecular signatures for virally-induced cancers programmatic focus.

Define molecular signatures through gene expression profiling of normal, precancerous and cancerous lesions (brute force screening). Gene expression profiling provides a powerful means for defining molecular signatures for diseases. Molecular signatures permit one to identify patterns of changes both at the RNA (gene chips/arrays) and protein (proteomics) levels between any given stage of cancer. They allow one to screen effectively for changes regardless of the level of knowledge that exists regarding the viral mechanisms of action in cancer. The success of this approach is based upon the availability and quality of the samples being compared. Human samples are optimal, where available; however, for many human cancers laboratory animal models or tissue culture models may be more practical sources of reproducible materials for analysis.

Cancer-associated viruses likely act, at least in part, in predisposing the cell to genetic changes, resulting in the progressive steps that lead to cancer growth. It may be beneficial, therefore, to identify early mutational changes in the progression to cancer. Such a genetic profile may be critical in identifying lesions with a high risk of developing cancers. While involving primarily a distinct set of technologies from that used in developing molecular signatures, the knowledge gained from generating a genetic profile of a cancer and its precancerous lesions may help predict the molecular signature for these lesions.

Define molecular signatures of virus induced cancers through an understanding of the mechanism of action of viruses. While gene expression profiling approaches may be beneficial, the availability of matched tissue samples or validated animal models may limit their application

utility. For many viruses, there is arising, through traditional reductionist scientific investigative efforts, fundamental insights into the mechanism by which they contribute to cancers. From these insights arise predictions for molecular signatures for virally-induced cancers. Thus, programs that test the validity of these predicted molecular signatures may provide great potential, especially where gene expression profiling studies are not feasible or as an augmentative approach to the latter.

Define host-immune responses that provide markers for progression of disease and susceptibility for disease. In all cases known, viruses that cause cancers are ones that persist for long periods in the host. How the host responds to the virus and how the virus modulates this response must be critical in allowing for viral persistence, and indeed may be determinative of the risk of cancer. Both the general immune competence and the type of immune response (e.g. type I versus type II) may be determinative of the outcome of the viral infection and subsequent risk of cancer development. In some cases, as with HBV and perhaps also HCV, cancer may result from an underlying immunopathological disease. Specific haplotypes may confer risk/protection to cancer; for instance, controversy surrounds assertions that haplotypes in class-II genes and extended haplotypes that include class-I-linked genes help determine risk of cervical cancer. In addition, oncogenic viruses in many cases have learned to evade the immune system; such evasion strategies likely contribute to their oncogenic potential. Therefore, the immunobiology of persistent viral infections and in particular those that lead to cancer must be better understood. This knowledge could contribute not only in helping identify patients at risk of developing cancer, but also in developing immunological intervention strategies.

Develop new/characterize existing animal models for virally-induced cancers. Validated laboratory animal models for virally-induced human cancers will be pivotal to the success of a molecular signatures program. For some cancers, laboratory animal models may be essential in providing the critical tissue samples with which to identify molecular signatures, not only for the frank cancer but more importantly the precancerous lesions that for some human cancers are difficult to identify/obtain. Animal models will also provide the means for testing the validity of a molecular signature, in understanding the underlying mechanism of action by which the viral agent contributes to the molecular signature, and in providing a means of testing new therapeutic modalities for intervening in virally induced cancers that might arise as a consequence of defining molecular signatures. Thus, a molecular signatures program may need to encompass the development and use of validated animal models for virally induced human cancers.