Presence of Simian Virus 40 DNA Sequences in Human Lymphoid and Hematopoietic Malignancies and Their Relationship to Aberrant Promoter Methylation of Multiple Genes

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Abstract

The simian polyoma virus SV40 has been detected in specific human tumors including non-Hodgkin’s lymphomas, although a causative role for the virus has not been convincingly demonstrated. Aberrant methylation of CpG islands in promoter regions is a frequent method of silencing tumor suppressor genes (TSGs) in cancers and may be induced by oncogenic viruses. We investigated the relationship between the presence of SV40 or EBV DNA sequences and the methylation profiles for 10 TSGs in 90 cases of non-Hodgkin’s lymphomas/leukemias and 56 control tissues. SV40 sequences were present in 33/90 (37%) non-Hodgkin’s lymphomas/leukemias, and EBV was present in 11/42 (26%) of non-Hodgkin’s lymphomas. We found a highly significant correlation between the presence of SV40 and methylation of seven genes (P values, 0.006 to <0.0001). In lymphomas, there was no relationship between EBV and methylation. Oncogenic viruses and methylation were rarely present in control tissues. We investigated methylation of the same 10 TSGs in peripheral blood mononuclear cells (PBMC) from a healthy volunteer infected with EBV or EBV and SV40. Promoter methylation of CDH1 and CDH3 were noted in dual SV40- and EBV-infected PBMC, and these two genes were also highly significantly correlated to the presence of SV40 sequences in tumors. SV40 infection also resulted in appearance of the lymphoma/leukemia-specific marker, methylated SHP1. Methylation was completely absent in uninfected and EBV-infected PBMC. Our results demonstrate that the presence of SV40 in hematological malignancies is associated with promoter methylation of TSGs and that in all probability, the virus plays a role in tumor pathogenesis.

Introduction

SV40 is a potent oncogenic virus of rhesus monkey origin and seems to have spread to human beings via contamination of poliovirus stocks between 1955 and 1963 as well as by other means (1). Inoculation of SV40 into hamsters results in the formation of mesotheliomas, brain tumors, bone tumors, lymphomas, and leukemias (reviewed in Ref. 2). Previous reports have found SV40 sequences in mesotheliomas, bone and brain tumors of human origin, lymphomas (reviewed in Ref. 2), and as demonstrated herein, we have recently extended these findings to leukemias. Although these observations indicate an association between the virus and specific human tumors, they do not demonstrate a causal role. Aberrant promoter methylation of CpG-rich areas of promoter regions is the most frequent mechanism of TSG silencing in human tumors (3). We have demonstrated previously that methylation of the TSG RASSF1A in malignant mesotheliomas is highly significantly associated with the presence of SV40 sequences (4, 5) and that SV40 infection of normal human mesothelial cells resulted in progressive methylation of the gene (5). For these reasons, we examined the methylation profile of 90 human lymphomas/leukemias, by testing aberrant methylation of 10 known or suspected TSGs and correlated the data with the presence of SV40 or EBV sequences.

Materials and Methods

Except for 19 specimens, personnel performing laboratory studies were blinded to the clinico-pathological diagnoses. Personnel performing viral assays (N. S. and V. S.) and methylation assays (T. T. and J. R.) were blinded to each other’s results. Additionally, the person (V. S.) performing real-time PCR analysis was blinded to results from conventional PCR analysis.

Tumors and Control Specimens. Tumors and tissues were obtained from the University of Texas Southwestern Medical Center affiliated hospitals, after receiving Institutional Review Board permission. They included 90 tumors (42 non-Hodgkin’s lymphomas and 48 leukemias). The lymphomas included 36 B-cell and 6 T-cell lymphomas; 7 were of high grade (Burkitt’s lymphoma) and 35 were of intermediate grade (diffuse large B cell, mantle cell, large cell anaplastic, follicular, and marginal zone). The leukemias consisted of 38 acute and 10 chronic of lymphoid or myeloid origin. Of the hematological malignancies, 62 samples had flow cytometric analysis that included determination of the percentage of tumor cells. The 56 nonmalignant tissue samples consisted of bone marrows (n = 10), peripheral blood (n = 42), and lymph nodes (n = 4) from healthy volunteers (n = 15), patients with nonmalignant hematological diseases (n = 11), or patients with hematological malignancies in remission (n = 30). The peripheral blood samples included three that were enriched for stem cells (0.5–1% stem cells).

Infection of Peripheral Blood Lymphocytes with EBV and SV40 Viruses. Peripheral blood mononuclear cells were isolated from a healthy volunteer and infected with EBV, SV40, or both viruses at a multiplicity of infection of 50–100 plaque forming units/cell (6). EBV virus stock was harvested from a chronically infected marmoset cell line (obtained from Dr. Nancy Schneider, University of Texas Southwestern Medical Center, Dallas, TX). SV40 virus stocks were obtained from Dr. M. Carbone (Loyola University Medical Center, Chicago, IL) as lysates from infected green African monkey kidney cells.

DNA Preparation and PCR Analyses. DNA was extracted (7) and analyzed for the presence of SV40 Tag sequences using primers that PCR amplified a specific 156-bp region of the large Tag of SV40 (8). Southern blotting and sequencing were performed on the resultant amplicons (Fig. 1). For analysis of EBV and SV40 sequences, real-time PCR assays based on TaqMan technology (Perkin-Elmer Corp., Foster City, CA) were used (9).

Serial dilutions of DNAs from an EBV-transformed human B lymphoblastoid culture and from a SV40-transformedhamster cell line (obtained from Dr. M. Carbone) were used to create a standard curve.

The sequences of primers and probes used to amplify and specifically detect EBV sequences were as described previously (10). β-Actin was used as an internal control for both assays. The sequences of primers and probes used to
of the two assays for SV40 Tag sequences (Fig. 2). The threshold cycle (C\(_T\)) values for the positive samples displayed a wide range, varying from 34 to 49. The SV40 hamster cell line had a C\(_T\) value of 26. The no template control and all 24 tumor samples negative by conventional PCR did not amplify at 50 cycles. Of particular interest, there was an excellent correlation (r = 0.904, P < 0.0001) between tumor cell percentage and C\(_T\) value.

EBV sequences were detected in 11 (26%) of 42 lymphoma samples (including all seven Burkitt lymphoma cases), in two (4%) of 48 leukemia samples (both from patients with chronic lymphatic leukemia) and in two (3%) of 56 control samples (both from lymphoma patients in remission). Both SV40 and EBV were present in 17% (7/42) of the lymphomas, including five Burkitt lymphomas and two diffuse large B-cell lymphomas. The possible relationship between Burkitt lymphoma, EBV, and SV40 needs to be explored further.

To examine whether there was any association between methylation and presence of SV40, frequencies of methylation for each gene in SV40-positive and SV40-negative samples were compared. The frequencies of methylation of the 10 genes in hematological malignancies varied from 19% for p73 to 97% for SHP1. For seven genes (CDH1, CDH13, p16, DcR1, DcR2, CRBP, and DAP kinase), the methylation frequencies were significantly higher in SV40-positive than SV40-negative cases, with P values ranging from 0.006 to <0.0001 (Fig. 3). There were no important differences between the methylation patterns of leukemias and lymphomas except that the differences between SV40-positive and -negative cases were statistically not significant for p16 and CRBP genes in leukemias and not significant for CDH1 in lymphomas (Fig. 3). Similar comparisons for EBV-positive and -negative lymphoma cases revealed no significant differences for any of the 10 genes studied (data not shown).

The mean MI (a reflection of the overall methylation frequencies for all genes tested) of SV40-positive cases was significantly higher than that of SV40-negative cases for all tumor cases and for lymphomas and leukemias individually (Fig. 3). Similar comparisons for EBV-positive (0.41 ± 0.05) and EBV-negative (0.33 ± 0.03) lymphoma cases revealed no significant differences for the mean MIs. SHP1, an inhibitor of the JAK/STAT pathway has been reported to be frequently methylated in lymphomas and leukemias. The SHP1 gene was methylated in 88 (97%) of the 90 hematological malignancies and in only one of the control samples (from a patient in remission). Of great interest, this sample was the only control sample positive for SV40.

Uninfected PBMC survived for only a few days, SV40-infected cells grew as adherent single cells having macrophage-like morphol-

**Results**

We found SV40 Tag sequences in 33 of 90 (36%) hematological cancers. With one exception (a leukemic patient in remission), we did not detect SV40 sequences in any of the 56 control samples (peripheral blood, bone marrows, and lymph nodes). In all cases, Southern blotting confirmed the specific nature of the detected amplicon. SV40 sequences were present in 17 of 42 (40%) non-Hodgkin’s lymphomas and 16 of 48 (30%) leukemias. The mean ages of SV40-positive and SV40-negative cancer patients were 51 (3–87) and 44 (neonate–80) years, respectively, and the differences were not statistically significant. Representative examples of SV40- and methylation-positive and -negative cases are illustrated in Fig. 1. Dilution experiments with leukemic samples indicated that at least 5% tumor cells were required for consistent, reproducible results.

Tumor cell percentage estimates (obtained by flow cytometric analysis) were available for 42 of the lymphoma/leukemia samples (18 of which were positive and 24 were negative for SV40 by conventional PCR-Southern blot analysis). Real-time PCR analysis of these 42 samples indicated complete concordance between the results and those obtained using the Southern blot method.
ogy for 14–16 days and then underwent lysis. EBV virus-infected cells, with or without the addition of SV40, grew as relatively rapidly dividing floating cells with uropod formation and exhibited morphology typical of EBV-transformed B cells (14). However, in the doubly infected cells, some attached macrophage-like cells also were present. After 6 weeks, both adherent and floating cells were harvested for molecular analyses. PCR-based assays indicated approximately equivalent amounts of EBV in single-infected (EBV) and dual-infected cultures. Real-time assays for SV40 indicated that the EBV virus-infected cells were negative for SV40 virus, whereas the dual-infected cells were positive, with a value (CT of 23) higher than that of the SV40-transformed hamster cell line (CT of 26).

MSP assays for methylation status of the 10 genes were performed on the EBV virus-infected and dual virus-infected cultures 6 weeks after infection. None of the genes was methylated in the uninfected or EBV-infected cultures. Real-time assays for SV40 indicated that the EBV virus-infected cells were negative for SV40 virus, whereas the dual-infected cells were positive, with a value (C_T of 23) higher than that of the SV40-transformed hamster cell line (C_T of 26).

MSP assays for methylation status of the 10 genes were performed on the EBV virus-infected and dual virus-infected cultures 6 weeks after infection. None of the genes was methylated in the uninfected or EBV-infected cultures. For the dual EBV- and SV40-infected cells, three of the 10 genes (CDH1, CDH13, and SHP1) were methylated 6 weeks after infection (Fig. 4).

Discussion

Previous studies from our laboratory and others (reviewed in Refs. 2 and 15) detected SV40 T-ag sequences in a subset of lymphomas. However, two recent studies from Europe have not supported these observations (16, 17). A recent meta analysis of the published literature supported the association between SV40 and certain human cancers including lymphomas (18). In the present study, we found viral sequences in 40% of lymphomas, compared with 43% in our previous study (19). We extended these findings in the present study and found SV40 Tag sequences in 30% of leukemias of both acute and chronic types. SV40 sequences, with one exception, were absent in marrow, blood, and lymph node samples from healthy volunteers, patients with nonmalignant hematological diseases, and patients with hematological malignancies in remission. For the SV40-positive cases, there was a 97% concordance with tumor cell presence (as detected by flow cytometry) and 100% concordance with methylation of SHP1, an almost universal marker for lymphomas and leukemias (20). In addition, there was complete concordance between the two PCR assays for T-ag and an excellent concordance between tumor cell percentage and CT value as detected by real-time PCR. These data are powerful evidence that the SV40 sequences are directly related to the presence of tumor cells.

Although we failed to detect SV40 in tissues from subjects without malignancy, it is possible that SV40 was present in nonmalignant tissues from patients with hematological cancers (and possibly in some of the control cases) but at levels that were not detected by our techniques. EBV sequences were, with rare exceptions, limited to lymphomas (26%) and were occasionally detected in subjects without malignancy.

Because previous reports suggest an association between aberrant methylation of TSGs and oncogenic viruses including SV40, we explored such a relationship in hematological cancers. Our previous studies indicated a relationship between the presence of SV40 and methylation of the RASSF1A gene in malignant mesotheliomas (4)
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and during transformation of mesothelial cells by the virus (5). In our present studies, we found a highly significant relationship between the presence of SV40 viral sequences and promoter methylation in hematological malignancies for seven of the 10 TSGs analyzed. Because SV40 was absent and the MI was low in controls, these changes were characteristic of tumors. Because statistical analysis showed no relationship between EBV and methylation in lymphoma/leukemia the relationship was limited to SV40, although EBV has been associated with increased methylation rates in gastric cancers (21). These findings indicate that EBV, may have different biological effects upon infection and transformation of different cell types. Although, the exact mechanism of aberrant methylation in SV40-positive cancers remains to be determined, the seven involved TSGs are located on five different chromosomal loci. Of interest, p16 and p15, which are located in tandem at chromosome 9p21, showed differential methylation, with p16, but not p15 being associated with SV40. Thus, SV40-associated hypermethylation is not a localized process restricted to one or a limited number of chromosomal loci but is a generalized process affecting CpG islands at multiple genomic sites. Promoter region methylation of multiple genes is a characteristic feature of all tumors (3). As SV40 appears to be neither necessary nor sufficient to induce cancer (2), methylation of some genes must also be present in tumors that are not virus associated. Thus, the virus-positive and -negative tumors had similar frequencies of methylation for three genes. These findings suggest that the methylation patterns of the two groups of tumors share certain similarities and differences, although our present study did not identify any genes whose methylation is characteristic of the virus-negative group.

Infection of PBMC with EBV resulted in lymphoblastoid transformation (14), whereas infection with SV40 resulted in the appearance of attached cells with macrophage morphology. These cells underwent lysis, which was complete by 6 weeks after infection. These findings suggest that SV40 infection resulted in productive infection rather than transformation (2). Dual infection with EBV and SV40 resulted in the appearance of a mixed population of nonadherent lymphoblastoid cells and adherent macrophage-like cells containing the sequences of both viruses. Of particular importance, promoter methylation of CDH1 and CDH13 was noted in dual SV40- and EBV-infected PBMC, and these two genes were highly significantly correlated to the presence of SV40 sequences in tumors. SV40 infection also resulted in methylation of the lymphoma/leukemia-specific marker SHP1. Methylation was completely absent in uninfected and EBV-infected PBMC, confirming the specific association between SV40 infection and methylation. Additionally, detection of methylation of SHP1 in SV40-positive EBV PBMC (although not in PBMC infected with EBV alone) supports the hypothesis that SV40 might be a causative agent in hematological malignancies. By an incompletely understood mechanism, DNA methyltransferases cooperate to catalyze overlapping but individualized genomic DNA methylation profiles of different cancer types (3, 22). A recent report indicates that transformation of a model system by SV40 and RAS resulted in expression of DNMT3b, which correlated with methylation and silencing of several TSGs (23). Expression of SV40 T-antigen increases histone acetylation and global histone acetyltransferase actions (24). Thus SV40 modulates both methylation and histone deacetylation, epigenetic processes that result in gene silencing. Our finding of a highly significant correlation between the presence of SV40 and aberrant methylation of multiple TSGs suggests that in all probability, the virus plays a role in tumor pathogenesis. These findings have considerable public health implications.

References

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![Fig. 4. Promoter methylation profile in EBV and EBV + SV40-infected PBMC. PBMC were infected with EBV or EBV + SV40 as described in “Materials and Methods.” MSP assays for 10 TSGs was performed as described in “Materials and Methods” (also see Fig. 1). The unmethylated form of p16 was run as an internal control for bisulfite treatment. The data show methylation of CDH1, CDH13, and SHP1 in EBV + SV40-infected group but none in only EBV-alone-infected group.](Image 69x600 to 271x749)
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