Expression of EBV Latent Antigens, Mammalian Target of Rapamycin, and Tumor Suppression Genes in EBV-Positive Smooth Muscle Tumors: Clinical and Therapeutic Implications

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Abstract

Purpose: EBV-positive smooth muscle tumor (EBV+SMT) is a rare disease with no established therapy. We describe the largest single institution analysis in renal transplant recipients. It aims to define its clinical features and determine the expression of EBV latent genes as well as key molecular pathways.

Experimental Design: Patients with EBV+SMT were identified from the Singapore General Hospital Renal Transplant Registry database. These tumors were investigated for expression of EBV latent genes with Southern blots, EBV latent antigens, mammalian target of rapamycin (mTOR), Akt, p70 S6 kinase, and vascular endothelial growth factor using immunohistochemistry, as well as methylation status of cancer-related genes using methylation-specific PCR.

Results: Eight were found to be EBV+SMT in 1,123 transplant patients. All displayed indolent clinical courses and were unresponsive to immunosuppression reduction. Complete tumor regression was seen in one patient following administration of sirolimus. These tumors display the full range of known EBV latent genes. Immunohistochemistry with total and phosphorylated mTOR and Akt were positive for all patients, and vascular endothelial growth factor was positive in 25% of patients, suggesting activation of the mTOR/Akt pathway. Methylation of RASSF1A was found in all tissue samples, whereas promoter hypermethylation of RARβ, GSTP1, DAPK, and p14 was observed in some samples.

Conclusions: Our results suggest that these tumors display a EBV type III latency pattern. The mTOR pathway is also activated. EBV may play a role in silencing RASSF1A. EBV-specific immunotherapy, mTOR inhibitors, and demethylating agents are possible therapeutic options in this disease. (Clin Cancer Res 2009;15(17):5350–8)

Prolonged immunodeficiency often results in the development of malignant tumors including de novo EBV-related neoplasms such as posttransplant lymphoproliferative disease (PTLD) and EBV-positive smooth muscle tumor (EBV+SMT). Reports on EBV-SMT in patients on immunosuppression following solid organ transplantation have been largely confined to case reports or small case series (1). We describe eight cases of EBV-associated smooth muscle tumors in patients on long-term immunosuppression following renal transplantation in the largest single-institution series documented.

Three types of EBV latent gene expression patterns have been well established in other EBV-associated malignancies (2). However, there is still much controversy, with regards to EBV expression pattern in EBV+SMT. Establishing the range of EBV latent gene expressions could potentially allow targeted immunotherapy against specific EBV latent antigens as with PTLD.

The mammalian target of rapamycin (mTOR) pathway plays an important role in regulating cell growth and proliferation and its deregulation is associated with many human cancers and diabetes (3). The mTOR pathway works by integrating various signals such as nutrient availability and growth factors to regulate different processes, including autophagocytosis, protein synthesis, and metabolism.

EBV+SMT is generally resistant to cytotoxic chemotherapy. Sirolimus inhibits mTOR and hence could be a potential therapeutic alternative for cancers for which the mTOR-Akt pathway is activated. It has been successfully used for the treatment of Kaposi’s sarcoma and, in our experience, with EBV+SMT (4, 5).
Characterization of EBV Smooth Muscle Tumors

Translational Relevance
EBV-associated smooth muscle tumors are rare malignancies known to occur exclusively in immunocompromised patients, typically as a result of post-transplant immunosuppression or HIV infection. However, most of the known literature have been restricted to case reports or small series. More importantly, little is known of its pathogenesis and therapeutic options. To the best of our knowledge, this article is the first paper to provide an in-depth analysis of the range of EBV latent gene expression and various molecular pathways that may be involved in its pathogenesis. These novel findings serve as a platform for development of novel therapeutic approaches for this condition, and potentially for other EBV-related malignancies.

Methylation of promoter regions reflect epigenetic changes that often occur in cancers, including those related to EBV oncogenesis [6]. Some common tumor suppressors including MGMT, DAPK, p14, and p16 and have been characterized for another EBV-associated malignancy, nasopharyngeal carcinoma (NPC; ref. 7). To provide further insight into EBV+SMT tumorigenesis, we did similar methylation studies to analyze their hypermethylation status. In this study, we studied the promoter hypermethylation status of seven genes, namely O6-methylguanine-DNA methyltransferase (MGMT), death-associated protein kinase (DAPK), retinoic receptor β gene (RARβ), ras association domain family 1A (RASSF1A), p14 (ARF), p16 (CDKN2A), and glutathione S-transferase (GSTP1). We showed the involvement of the mTOR pathway in the oncogenesis of EBV+SMT with immunohistochemistry. This study represents the most comprehensive clinical, histopathologic, and molecular study of EBV+SMT and their potential therapeutic implications in the largest single-institution series to date.

Patients and Methods

Patients and tissue samples. We identified patients with histologically proven EBV+SMT from the Singapore General Hospital Renal Transplant Registry database between 1989 and 2004. These patients were all on long-term immunosuppression after renal transplantation. Additional clinical information was obtained from case records. Available frozen tissues from five patients with EBV+SMT were used. Controls included six EBV-negative leiomyosarcomas from immunocompetent patients (EBV-SMT). These tissues were obtained from the National Cancer Centre, tissue repository, Singapore, with prior patient consent. EBV-positive tissues were also paraffin embedded and subsequently used for immunohistochemical analysis.

Cell lines. The C666-1 cell line is an EBV-positive NPC cell line, which was kindly provided by Dr. Kwok Wai Lo (Chinese University of Hong Kong, SAR, China). It was maintained in RPMI 1640, 10% FCS, and 2 mmol/L l-glutamine. CNE1 and CNE2 cell lines, both EBV-negative NPC cell lines, were kindly provided by Prof. Kam Man Hui (National Cancer Centre, Singapore) and maintained in DMEM, 10% FCS, and 2 mmol/L l-glutamine. Raji (an EBV-positive BL line), and B95-8 (a mammosot cell line carrying infectious mononucleosis-derived EBV) were kindly provided by the WHO Immunology Center, Singapore. They were maintained in RPMI 1640 and 10% FCS.

In situ hybridization. Presence of EBV-associated small RNA (EBER) was determined in tumor tissues from all eight patients using the EBER probe (DakoCytomation), which was used in conjunction with the DakoCytomation PNA in situ hybridization (ISH) Detection kit as previously described [8].

Southern blot. Total RNA was extracted using RNeasy Mini kit (Qiagen) from patient frozen samples. Detailed sequences of primers and probes have been described before [9-11]. The probes were labeled with DIG-dUTP by DIG oligonucleotide 3′-end labeling kit (Roche Diagnostics). Approximately 1.5 μg RNA of EBV-positive samples were subjected to cDNA synthesis with a reverse transcription system (Applied Biosystems), and PCR was carried out using 1X AmpliqGold (Applied Biosystems) in a total reaction volume of 20 μL. Annealing temperatures have been described as before. The amplified products were electrophoresed in 2% agarose gel, transferred onto a Hybond N+ nylon membrane (Amersham Pharmacia Biotec), and subjected to hybridization with 3′-end-DIG-labeled oligonucleotide probes. The hybridized signals were detected by alkaline phosphatase-conjugated anti-DIG antibodies. The substrate of alkaline phosphatase was CSPD (Roche Diagnostics). cDNAs from EBV-immortalized lymphoblastoid cell lines were used as positive controls, and those from EBV-negative PTLDs as negative controls. The integrity of RNA was checked by amplification of glyceraldehyde-3-phosphate dehydrogenase mRNA.

Immunohistochemistry. Immunohistochemistry was done on formalin-fixed paraffin-embedded sections from seven patients, using the peroxidase detection system (EnVision®, DakoCytomation). Slides were counterstained with hematoxylin. Antibodies against EBNA2 (dilution, 1:50), desmin (dilution, 1:100), CD21 (dilution, 1:25), Bel-2 (dilution, 1:100), Ki67 (dilution, 1:200), LMP-1 (dilution, 1:100), and SMA (dilution, 1:200) were from DakoCytomation. Antibodies to total mTOR (dilution, 1:30), phospho-mTOR (Ser2448, dilution, 1:20), total p70 S6 Kinase (dilution, 1:60), phospho-S6K (Thr389, dilution, 1:20), Akt1 (dilution, 1:20), and phospho-Akt (Ser473, dilution, 1:10) were purchased from Cell Signaling Technology. Anti–vascular endothelial growth factor (VEGF; dilution, 1:20) was purchased from R&D Systems.

Bisulfite modification and methylation-specific PCR. The methylation status of MGMT, DAPK, RARβ2, RASSF1A, p14, p16, and GSTP1 were assessed by methylation-specific PCR as described previously (7). Genomic DNA from primary tumors, cell lines, and normal smooth muscle tissue were modified by bisulfite treatment and purified using the CpGenome Modification kit (Chemicon) as recommended.

Results

Clinical features. A total of 104 distinct tumors were identified in 1,123 renal allograft recipients on follow-up at our institution from 1989 to 2004. Of these, eight patients with EBV+SMT were identified. All five males and three females were ethnic Chinese adults, with ages ranging from 29 to 61 years (mean, 42.3 years). Patient characteristics are listed in Table 1. The time interval between renal transplantation and tumor development varied from 2 to 13 years (mean, 6.1 years). Seven of the eight patients are still alive, with a mean survival time of 67.6 months. Of these, only two patients remained disease free. The cause of death in patient four was sepsis secondary to nosocomial pneumonia and urinary tract infection rather than from disseminated EBV+SMT. All cases except patient two had reduction of immunosuppression, but without any evidence of tumor regression. Although six patients underwent surgery, only patients three and five achieved complete tumor resection. Based on our records, there were no unique clinical features in these eight patients either before or posttransplant.
Six of the eight patients had tumors in multiple sites based on radiological or histologic evidence. Only two patients had single site involvement, both confined to the liver. The majority of the cases (n = 7) had tumors in the lung or liver, or both. Unusual sites of presentation included skin, bone, adrenal, and the upper aerodigestive tract. Four of eight patients were symptomatic at presentation, whereas the rest had tumors detected incidentally on radiological evaluation. EBV+SMT accounted for 7.7% of all 104 neoplasms in 1,123 patients following renal transplantation. The incidence of these tumors was nearly equal to the well-described EBV-associated tumor common in immunosuppressed patients, PTLD, for which 10 cases (9.6%) were diagnosed during the same period, in the same cohort, in our institution.

EBV capsid antigen IgG, but not IgM, was positive in all eight patients at the time of diagnosis, indicating previous rather than recent EBV infection. Corresponding cell-free plasma EBV DNA levels was analyzed for these patients except patient 4. These

<table>
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<th>Patient</th>
<th>Sex</th>
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<th>Ethnicity</th>
<th>Time to tumor dx (y)</th>
<th>Survival (mo)</th>
<th>Sites</th>
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<th>Treatment</th>
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Abbreviation: Dx, diagnosis.

Table 1. Patient demographics

Fig. 1. A, H&E staining of EBV+SMT found in the glottis of patient 1, which stains positively for SMA (B). C, EBER was present on ISH in EBV+SMT. D, strong immunohistochemical staining for EBNA2. Magnification, ×40.
ranged from 0 to 109 copies/mL, which are within the reference range and not elevated.

Our center uses a calcineurin inhibitor–based regimen for all renal transplant patients. All eight patients were on cyclosporin and prednisolone at time of diagnosis. In addition, patients 3, 4, 6, 7, and 8 were receiving azathioprine, whereas patients 1 and 2 were on mycophenolate mofetil. Patient 2 with EBV+SMT in her liver was treated with sirolimus, resulting in complete remission of the tumor as previously described (5). Her initial immunosuppression regimen consisted of 75 mg cyclosporin twice a day, 8 mg prednisolone daily, and 500 mg mycophenolate mofetil twice a day. Upon diagnosis, immunosuppression was reduced to 60 mg cyclosporin twice a day and 7 mg prednisolone daily, with complete withdrawal of mycophenolate mofetil. Due to tumor progression on follow-up imaging 3 mo after initial diagnosis and concomitant cyclosporin-induced arteriopathy, cyclosporin was tapered off over 2 months and sirolimus was simultaneously started. After a loading dose of 10 mg, sirolimus doses were titrated to achieve a trough concentration of 5 to 12 ng/mL with final maintenance dose of 2 mg. Her tumors regressed, and to date, she remains in complete remission.

**EBV gene expression.** Figure 1A shows a typical EBV+SMT found in the glottis of patient 1, consisting of fascicles of elongated and rounded cells with scattered lymphocytes. Staining for smooth muscle actin (SMA; Fig. 1B) and desmin was positive for all samples. Tumor cells showed strong diffuse staining for EBER on ISH, confirming EBV positivity (Fig. 1C).

Table 2A and B summarize the immunostaining and ISH results. Samples were scored as negative when no immunoreactive tumor cells were observed. Positive samples were semiquantitatively categorized into three groups according to the percentage of immunoreactive tumor cells: 1+ representing <10%, 2+ representing 10 to 50%, and 3+ representing >50%. Proliferation index (Ki-67 expression) was low and BCL-2 was largely absent, whereas EBNA2 was present in tumors from all six patients on immunohistochemistry (Fig. 1D). LMP1 and the receptor for EBV, CD21, were also absent in all samples.

Five EBV+SMT were available for analysis of EBV gene expression (Fig. 2). The promoter Cp was activated in all tumors. Wp was activated in two of the five tumors, whereas Qp were activated in only patient 6. EBV latent antigens EBNA1, 3A, and B were expressed in all EBV+SMT tested, whereas expression of EBNA2 and LMP2 were occasionally seen. As with immunohistochemistry, LMP1 was not detected. Both EBV lytic antigens BZLF and BRLF were not expressed. As expected, all six smooth muscle tumors from immunocompetent patients were tested negative for EBV genes.

**Involvement of the mTOR pathway.** Cytoplasmic staining for total Akt (Fig. 3A) was very strong, with the exception of patient 3 and 7, where focal staining was observed (Table 2B). Phosphorylated Akt showed moderate staining intensity (Fig. 3B) in the nucleus and cytoplasm for at least 80% of the patient samples. Corresponding total mTOR and its phosphorylated form were positive for all patients (Fig. 3C and D). Our results of mTOR

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**Table 2. Immunostaining and ISH results**

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<th>A. Immunostaining and ISH results of the tumor cells from patients</th>
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<th>EBNA2</th>
<th>Ki-67</th>
<th>CD21</th>
<th>LMP1</th>
<th>Bcl-2</th>
<th>Desmin</th>
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<th>B. Immunostaining results for VEGF, total, and phosphorylated forms of Akt, mTOR, and p70S6 kinase</th>
<th>Patient</th>
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Abbreviations: SMA1A4, smooth muscle actin; EBNA2,: Epstein-Barr nuclear antigen-2; CD21, type II complement receptor CR2; LMP, latent membrane protein; EBER-ISH, EBV-encoded small RNA-ISH; -, negative staining (1+, weak staining with <10% positive cells; 2+, moderate staining with <50% positive cells; 3+, strong staining with >50% positivity); phos-, phosphorylated; N/A, not applicable; focal, staining of <20% tumor cells.
are consistent with previous observations of strong cytoplasmic staining for total mTOR in primary liver neoplasm compared with normal liver controls (6). Staining intensity for total and phosphorylated Akt, as well as total and phosphorylated mTOR, were all weak in EBV-SMT (Supplementary Figures). Results for total p70 S6 kinase showed intense focal staining for all patients studied, whereas phosphorylated p70 S6 kinase was also positive with 66% of the samples showing very strong nuclear staining (Supplementary Figures). Moderate to high focal expression of VEGF (staining for only <20% tumor cells) was shown in only 25% of the patient samples (Table 2B). Normal controls were also done using myometrium tissue. Staining intensity was negative for all phosphorylated proteins. Total Akt and total p70 S6 kinase had moderate staining intensity of 1+ only.

**Methylation of gene promoters in patient samples.** The methylation-specific PCR analysis results of the cases are presented in Fig. 4. Our controls showed results which are consistent with previous reports. All patient samples were methylated for RASSF1A. EBV-negative leiomyosarcoma samples were only methylated in two of five samples. No methylation of MGMT was detected in all tissue samples obtained from five patients. GSTP1 and p14 were hypermethylated for patients 1, 3, and 5. RARβ and DAPK had similar results with hypermethylation present in patients 1 and 3 only.

**Discussion**

Our series confirms that these smooth muscle tumors are EBV related (eight of eight were EBER positive) and has similar histopathology characteristics to previous reports (12). All our patients were Chinese, suggesting an ethnic predisposition to the tumor as our study population, although predominantly Chinese, included patients of races such as Malays and Indians. In contrast to a predominantly Western paediatric population described in previous reports, all of our patients were adults. Our results indicate that the disease does not exhibit an age-specific predilection. Rather, the likelihood of developing of EBV+SMT may be related to the degree of immunosuppression induced.

EBV+SMT may arise long after renal transplant (from 24-156 months), which is consistent with a previous report (13). These tumors were indolent in behavior, even in advanced, multifocal disease. Seven of our patients are still alive, five with radiological evidence of slowly progressive disease. The low expression of the proliferation marker Ki-67, together with the absence of a major antiapoptotic protein Bcl-2, in our tumor specimens supports its biological behavior.

Tapering of immunosuppression did not cause tumor regression in any of our patients, although there have been reports of...
EBV+SMT tumor regression following reduction of immunosuppression (14, 15). It is plausible that tapering of immunosuppression may slow down EBV+SMT tumor kinetics by improving immunity (15, 16). Because of historically reported poor response to chemotherapy in EBV+SMT, we did not treat any of our patients with cytotoxic chemotherapy (14, 15). Curative surgery should be advocated whenever possible (13). However, this is usually not possible as evidenced in our patients due to the multifocal nature of this condition.

Our data confirms that EBV+SMT are frequently multifocal and can be found in unusual sites (17–20). Liver is the commonest site of involvement (n = 6) as previously reported (19), with the propensity for liver involvement is especially common in posttransplantation patients (15).

EBV+SMT in our series expectedly stained strongly positive for EBER and SMA. The EBV receptor, CD21, was negative in all eight patient samples, consistent with previous reports of EBV+SMT in organ transplant patients (19, 21), with some exceptions (14, 22). CD21 is also not expressed in another EBV-related epithelial tumor common in southern Chinese, NPC (23). Thus, tropism of EBV for epithelial cells may occur via CD21-independent mechanisms and pathways (24).

All eight patients showed previous EBV infection based on serologic tests. However, as this is a retrospective study, exact titers of these antibodies were not done in our patients before and after diagnosis. Hence, we are unable to rule out the possibility of EBV reactivation. Unlike the case in NPC, none of the patients showed a markedly elevated cell-free plasma EBV DNA levels (25). This precludes the use of quantitative analysis of EBV DNA levels as a screening or surveillance tool.

The pattern of EBV latency genes in EBV+SMT remains controversial. In an earlier report, EBV expression in these tumors was thought to follow a type III pattern in which the full array of EBV latent genes is expressed (19). However, this has been disputed by other reports where a latency type I pattern was found instead, i.e., presence of EBNA1 and both EBNA2 and LMP1 undetectable (17, 26). In our series, the EBV latent gene expression in EBV+SMT seems to be most consistent with the type III latency pattern. This latency pattern can only exist during the acute phase of primary infection and in patients with impaired immunity, as is the case with our transplant patients who are on immunosuppressive agents (27). Although we acknowledge the lack of consistency in the expression of EBNA2 and LMP1 in all EBV+SMT tumors, this is likely due to the inadequate sensitivity of our assay rather than artifactual findings. Indeed, LMP1 was also undetected in previous studies, raising the possibility that the amount of LMP1 expressed is below the threshold of detection (14, 16, 18). Our findings of a type III latency pattern are further supported by the consistent activation of Cp and in some cases, Wp promoters. These promoters are activated in type III latency resulting in expression of all EBNA2 in all tumor samples (2). Additionally, Qp that is normally activated in the more restrictive types I and II latency patterns seen in various non-B-cell tumors resulting in expression of only EBNA1, is predominantly silenced in our samples. In the single tumor specimen in which Qp was activated, both Cp and Wp were also strongly expressed, with a resultant type III latency pattern seen.

Our findings of a type III latency gene expression pattern in a tumor of smooth muscle origin is highly significant, as it is thought that expression of EBNA-2, 3, and LP requires specific transcription factors, which are present only in B lymphocytes (2). The expression of EBNA2 other that EBNA1 in these tumors in the absence of essential B cell-specific transcription factors

Fig. 3. A, total Akt, (B) phosphorylated Akt, (C) total mTOR, and (D) phosphorylated mTOR. Magnification, x40.
Fig. 4. Methylation-specific PCR analysis of p14, p16, RASSF1A, RARβ2, MGMT, GSTP1, and DAPK expression in NPC cell lines (CNE1, CNE2, C666-1), EBV-positive cell lines (Raji, B95-8), patient biopsies (patient 1, 3, 4, 5, and 7), and EBV-negative tumor samples. PCR products in lane u indicate presence of unmethylated templates of each gene, and lane m indicate presence of methylated templates. H2O, water control; N, adjacent normal; T, primary tumor.
such as BSAP and Oct-2 suggests that either expression of EBNA proteins in smooth muscles are independent of these factors, or that homologues of these factors may be present in these cells.

The expression of the full range of known EBV latent genes lends itself to the potential of the use of viral antigen-specific immunotherapy, as seen with adoptive transfer of EBV-specific CTLs in PTLDs (28). The significance of such an approach is amplified by the paucity of effective therapeutic options currently available for EBV+SMT.

In addition to adoptive cell-based immunotherapy, we have described early clinical data on sirolimus as a potential antineoplastic agent for this rare tumor (5). Patient 2 achieved complete resolution within 5 months of starting the therapy that has sustained remission over 42 months to date.

EBV is a pathogen that is able to escape immune surveillance during its latent stage and, upon reactivation, cause various malignancies such as NPC, PTLDs, and Burkitt's lymphoma (29). Reports have shown that it survives in its host undetected and activates the phosphoinositide 3-kinase/Akt pathway, which plays a key role in cell proliferation (30). Expression of mTOR was consistently high in our patient cohort. Downstream proteins involved in mRNA translation such as phosphorylated forms of mTOR and p70 S6 kinase expression were also highly expressed in most patients. The EBV+SMT patient who responded to sirolimus with a sustained complete remission had moderate expression of total mTOR, and phosphorylated Akt, but only weak expression of phosphorylated mTOR (5). Our immunohistochemical data supports the notion that sirolimus may be acting through the rapamycin-associated protein (FRAP/RAFT/RAFT/SEP) pathway as an inhibitor of mTOR (FKBP12; ref.. 31, 32).

Little is known about the function of mTOR and initial understanding included its involvement in protein synthesis through p70 S6 kinase, which regulates gene translation (33). The current knowledge of how mTOR functions includes the existence of two distinct multiprotein complexes involving either the raptor or rictor protein (34, 35). Previous data have shown that cancer cells with disrupted PTEN function and overexpressed Akt/PKB are highly sensitive to the antiproliferative effects of sirolimus (36). Sirolimus has been shown to be involved only with the raptor complex, where it acts to weaken the raptor-mTOR interaction (37). On the other hand, the rictor-mTOR complex is a substrate of the Akt signaling pathway, which is often active in cancer cells (38). Our results showed increased staining intensity of phosphorylated Akt and p70 S6 kinase compared with normal smooth muscle tissue, consistent with a recent report (39). It is possible that prolonged treatment of sirolimus may inhibit the rictor-mTOR complex to directly suppress the Akt signaling pathway, thereby reducing tumor growth. This is corroborated by the use of sirolimus for at least 5 months before tumor size was observed to decrease in a patient with EBV+SMT (5). Thus, sirolimus may have a two-pronged effect: influencing cell growth through protein synthesis and cell survival and proliferation through the Akt pathway. The role of EBV in influencing the phosphoinositide 3-kinase/Akt pathway was shown in a recent study, where latency protein LMP2A activated the phosphoinositide 3-kinase/Akt and mTOR pathways, thus providing evidence that the activation of phosphoinositide 3-kinase/Akt pathway in EBV-related tumors is responsible for the sensitivity of the tumor to mTOR inhibitors (40).

Epigenetic alterations have long been suspected to be involved in EBV-related disease development. Because EBV infection is closely associated with NPC, which is endemic in this region, we decided to study the methylation status of some tumor suppressor genes that have already been analyzed in NPC. Consistent with similar reports in NPC samples, we found RASSF1A, a protein that stabilizes microtubules and regulates mitotic events, to be methylated and MGMT, a gene that allows DNA repair, and protects it from environmental alkylating carcinogens (41), to be unmethylated for all patient samples. Similar reports have suggested that latent infection of EBV may cause a loss of gene function for RASSF1A, which may be involved in the transformation and development of EBV+SMT. Previous studies have reported MGMT gene to be thrice more likely to be methylated in EBV+ compared with EBV-gastric cancer (42), yet only methylated in 20% of NPC in another series (7). The p16 gene, an inhibitor of the G1-S transition of the cell cycle, was also observed to be unmethylated for all samples except for patient 3, compared with EBV-negative samples, which were predominantly hypermethylated. Another cell cycle regulatory protein, p14, was found to be hypermethylated for patient 1, 3, and 5. Our data suggest that inactivation of p14 and p16 may have little or no importance in the development of EBV+SMT. A recent study has provided evidence that LMP1 down-regulates RASSF1A expression, facilitating tumorigenesis (43). As mentioned earlier, Southern blot analysis in our study showed an absence of LMP1 expression. A possible reason may include a different mechanism of dysregulation of RASSF1A precluding the involvement of LMP1.

DAPK is a positive mediator of apoptosis induced by IFN-γ (44). It may be inactivated by methylation in promoter regions of the gene in cancers such as NPC (45). Our study detected methylation of DAPK in both patients 1 and 3. GSTP1 play an important role in detoxification and protect cells from carcinogens. Hypermethylation of GSTP1 was observed only in patient 1, 3, and 5. Silencing of DAPK and GSTP1 may not be a biologically significant event in EBV+SMT. In contrast, hypermethylation of GSTP1 was detected in all EBV-negative leiomyosarcoma samples. Retinoic acid receptor mediates the effects of retinoic acid, a vitamin A metabolite, allowing cell proliferation and differentiation (46). Previous reports have shown that silencing of RARβ2 is common in NPC cell lines and tumors (7). Our study detected methylation in patients 1 and 3 only. A recent article by Kwong et al. (47) has shown that retinoid signaling molecules (such as cellular retinoid binding protein and cellular retinoid acid binding protein) are also hypermethylated and may contribute to the disruption of retinoid signaling pathway in this cancer, resulting in the unresponsiveness of patients to retinoic acid treatment.

With the exception of MGMT, DAPK, and p16, our results illustrate that the CpG hypermethylation status in EBV-related tumors may allow the possibility of therapeutic intervention with demethylating agent, azacitadine, which may activate silenced viral genes EBV+ tumor and induce a potential immune-mediated destruction of EBV+ tumor cells (48). Our results show that of seven tumor suppressor genes, hypermethylation of the RASSF1A promoter is a common event in EBV-SMT, illustrating its potential as a clinical marker for the disease.

In summary, we believe that this study represents the most extensive attempt to characterize EBV+SMT to date. In addition to its clinical features, we have provided data on the pattern of EBV latency gene expression as well as molecular and methylation studies. Based on our results, we conclude that EBV-based immunotherapy, mTOR inhibitors, as well as demethylating agents
against EBV-SMT and other EBV-transformed tumors warrant further examination.

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We confirm that this study was done with informed consent from the patients concerned.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

References
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