Identification of Loss of p16 Expression and Upregulation of MDR-1 as Genetic Events Resulting from Two Novel Chromosomal Translocations Found in a Plasmablastic Lymphoma of the Uterus

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Abstract

Purpose: To establish cell lines from the patient with plasmablastic lymphoma, who was immunologically competent including negative human immunodeficiency virus (HIV) serology, and analyze the unique chromosomal translocations seen in the cell lines in order to unveil the pathogenesis of this tumor, which had no evidence of Epstein-Barr virus involvement.

Experimental Design: Establishment of the cell lines was attempted by inoculating the patient’s lymph node biopsy specimen subcutaneously to immunodeficient mice. Comparative genomic hybridization (CGH) array and FISH analysis were performed to identify breakpoints of the two chromosomal translocations. Of the 4 candidate genes identified by FISH analysis to be involved in the translocations, reverse transcription-PCR, Western blot, flow cytometry, and proliferation assay were performed to identify the exact genes involved.

Results: Analysis of the cell lines identified loss of p16 at the protein level by chromosomal translocation of t(9;13) and upregulation of MDR-1 by t(4;7). The cell lines expressing MDR-1 acquired resistance to chemotherapeutic agents such as cisplatin and doxorubicin, but not bortezomib. Expression of B lymphoid lineage marker genes of these cell lines was negative for paired box 5 (Pax5) or PR domain containing 1, with ZNF domain (PRDM1), but was positive for X-box binding protein 1 (Xbp1).

Conclusions: We established three novel cell lines of plasmablastic lymphoma. Characterization of the unique chromosomal translocation identified loss of p16 and upregulation of MDR-1 at protein level. Expression of Xbp1(s), which is involved in the maturation of plasma cells, corresponded to the plasmablastic appearance of the tumor. These cell lines may be a useful tool to understand the pathophysiology of the disease and to develop novel treatment strategies.

Introduction

Plasmablastic lymphoma was first reported by Delecluse and colleagues (1) in 1997 as a variant of diffuse large B-cell lymphoma (DLBCL). In the recently revised World Health Organization classification, it has been classified as a distinct clinical entity of DLBCL (2). Histologically, it is characterized by diffuse proliferation of large neoplastic cells with immunophenotype of plasma cells. It is most frequently seen as a mass in the oral cavity, but it may also be encountered in other extranodal sites including the sinonasal cavity, orbit, skin, bone, soft tissues, and gastrointestinal tract (2). Immunodeficiency, mainly by human immunodeficiency virus (HIV), is the major risk factor for this disease; however, some cases present without any history of immunodeficiency (3–5). Most patients present at an advanced stage with the international prognostic index (IPI) in the intermediate or high risk score. It is clinically aggressive with a median survival of 6 months, with most patients dying within 1 year of the onset of disease; however, it has been suggested that the addition of highly active antiretroviral therapy (HAART) to chemotherapy is capable of significantly improving the prognosis in HIV-positive patients (2, 5, 6). Epstein-Barr virus (EBV) or Kaposi sarcoma–associated human herpesvirus 8 (HHV8) have been proposed to play a relevant role in the pathogenesis of this disease, although the involvement of the latter remains controversial (7–10). Altogether, the pathogenesis of plasmablastic lymphoma is yet largely unknown.

Note: Supplementary data for this article are available at Clinical Cancer Research Online (http://clincancerres.aacrjournals.org/).

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The pathological findings of the resected uterus showed proliferation of anaplastic large cells with deposition in the cytoplasm and deviated nucleus, suggesting a plasma cell neoplasm. The cells were positive for plasma cell markers EMA, CD138, and VS38c, and were negative for various epithelial markers and immunoglobulin by immunostaining (Fig. 1B and data not shown). She was referred to our service for the treatment of highly anaplastic plasma cell tumor. Bone marrow and spinal fluid examination showed no infiltration of tumor cells. Lytic bone lesions were not evident on skeletal X-ray. Soon after the referral, she developed altered mental status. Laboratory findings showed hypercalcemia, and she was put on dialysis and nephrostomy tube was placed because of hydronephrosis due to multiple paraaortic lymph node swelling. Considering her pathological diagnosis and clinical course, she was diagnosed as having plasmablastic lymphoma. After her general condition was stabilized, she was treated with cyclophosphamide, DXR, vincristine, and prednisolone (CHOP). However, the disease progressed shortly after the treatment. She was treated with hyperfractionated cyclophosphamide, vincristine, DXR, and dexamethasone (hyper CVAD) therapy (11), and subsequently placed on vincristine, DXR, dexamethasone (VAD) therapy (12) on day 13 of hyper CVAD regimen. The chemotherapies produced minimal effect, and she died of progressive disease on day 7 of VAD therapy.

Establishment of cell lines
Lymph node specimen from the patient with plasmablastic lymphoma was inoculated subcutaneously to immunodeficient mice, first to NOD/Shi-scid, IL-2Rγnull (NOD) mice (13) and subsequently to nude mice. The lymph node was simultaneously dispersed and cultured in RPMI1640 medium (Sigma-Aldrich) supplemented with 10% FBS. The subcutaneous tumor from NOD mice was cultured in RPMI1640 medium or in Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% FBS. Karyotype analysis and Southern blot analysis of the established cell line was performed at an outside laboratory (BML Inc.). Immunophenotyping using flow cytometry was performed at the clinical laboratory of our hospital. Ultrastructure of the cell lines was analyzed by electron microscopy as previously described (14).

Comparative genomic hybridization analysis
Comparative genomic hybridization (CGH) array made by NimbleGen (currently Roche NimbleGen) that covers the entire regions of chromosome regions 4q35, 7q22, 9p22, 13q22 with 5-Mbp margin on both ends was used. The probes were 50 to 75 mer in length, and designed 100 bp apart. Whole genomic DNA was extracted from the established cell line using Gentra Systems Puregene genomic DNA purification kit (Qiagen). Human genomic DNA from multiple anonymous donors (female; Promega Corporation) was used as control template. Analysis of CGH array was performed by Roche Diagnostics KK.

Fluorescence in situ hybridization analysis
Bacterial artificial chromosome (BAC) clones corresponding to the regions 4q35, 7q22, 9p22, 13q22 were selected from Keio BAC library (15) every 3 Mbp and...
FISH probes from these selected clones were prepared at GSP Laboratory (Kanagawa, Japan). Preparation of samples and FISH analysis were performed as previously described (16).

**Antibodies**

FITC-labeled anti-human MDR-1 antibody and PE-labeled anti-human CD138 antibody were products of BD Pharmingen. Mouse anti-p14 antibody (14P01) was a product of Thermo Fisher Scientific and both rabbit anti-p15 antibody (sc-612) and rabbit anti-p16 antibody (sc-468) were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-β-actin antibody was purchased from Cell Signaling Technology, Inc.

**Analysis of expression and function of MDR by flow cytometry**

The expression of MDR protein on the cell lines was analyzed with fluorescence-conjugated monoclonal antibody using EPICS-XL flow cytometer (Beckman Coulter). The function of MDR protein was analyzed by measuring the exclusion of rhodamine 123 dye by flow cytometry (17).

**Western blot analysis**

The protein expression of p14, p15, p16, and β-actin was analyzed by SDS-PAGE and Western blotting. Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG antibody or HRP-conjugated donkey anti-rabbit IgG antibody (GE Healthcare) were used as secondary antibodies. The proteins were detected using ECL [enhanced chemiluminescence (Amersham)] Western Blotting Detection Reagents and Hyper ECL film (GE Healthcare).

**Reverse transcription-PCR**

Total RNA was extracted using RNaseasy Mini Extraction Kit (Qiagen). An amount of 0.5 μg of total RNA was subjected to reverse transcription using SuperScript First-Strand Synthesis System (Invitrogen) and PCR as per manufacturer’s protocol. Expression of the target genes was assessed using primer pairs described in Supplementary Table S2. Takara Ex Taq Hot Start version (Takara Bio Inc.) was used for PCR amplification. HeLa cells were used as positive control for p14, p15, and p16 analysis. RPM8226, Daudi, U266 cells were used as a positive control for X-box binding protein 1 (Xbp1), paired box 5 (Pax5), and PR domain containing 1, with ZNF domain (PRDM1) analysis, respectively.

**Sequence analysis**

Reverse transcription-PCR and PCR amplification of the coding region of p16 mRNA was performed as previously described using the primer as noted in Supplementary Table S2. The product was sequenced using BigDye terminator cyclesequencing kit (Applied Biosystems) and an automated sequencer, ABI Prism 310 genetic analyzer (Applied Biosystems).

**Proliferation assay**

Cells were cultured at a density of $1 \times 10^4$ cells/200 μL in 96-well plates with various concentrations of cisplatin, doxorubicin, or bortezomib. Ten microliters of WST-8 reagent (Kishida Chemical) was added to each well during the last 4 hours of culture. Absorbance was measured at a wavelength of 450 nm using 96-well microplate reader, Model 680 (Bio-Rad). Each experiment was performed in triplicate and 3 independent experiments were performed.

**Ethical issues**

Research protocol was approved by the ethics committee of our institute and written informed consent was obtained from the patient.

**Results**

Cervical lymph node biopsy was performed before the initiation of VAD therapy, in order to confirm the diagnosis and evaluate the cause of highly aggressive nature of the current tumor. Histologically, the findings were identical to its original tumor at the uterus. The karyotype of the tumor subsequently narrowed down (Supplementary Table S1).
cells showed either 46,XX, t(9;13)(p22;q22); 46,XX, t(4;7) (q35;q22), t (9;13)(p22;q22) or 46,X, add (X)(p22), t (9;13)(p22;q22) in 3, 16, and 1 out of 20 metaphases analyzed, respectively. Immunoglobulin (Ig) heavy chain rearrangement and the genomic integration of c-myc and EBV were negative by Southern blot analysis (data not shown). HHV8 status was not examined in our specimen.

The lymph node specimen was subcutaneously inoculated to NOG mice that produced a mass histologically identical to the original tumor (Fig. 2A). The tumor was subsequently transplanted to nude mice that showed stable engraftment, and the tumor was stably transplanted up to 5 passages.

Altogether 3 cell lines were cloned and stably cultured in vitro, 1 from the original patient tumor from the lymph node and 2 from the once established tumor cell line in NOG mouse. We named these cell lines KY-1, 2, and 3, respectively. Morphologically, the tumor cell lines showed anaplastic plasmacytic appearance (Fig. 2B). Immunophenotype of all 3 cell lines was identical to the original tumor. They were positive for CD138 and negative for other lineage markers, CD3, 5, 10, 14, 16, 19, 20, 22, 23, 38, 45, 56 (Fig. 2C and data not shown) on flow cytometry. Karyotypes of each cell line were as follows: KY-1: 46, XX, t(9;13)(p22;q22), del(20)(q12q13); KY-2: 46, XX, t(4;7)(q35;q22), t(9;13)(p22;q22); KY-3: 46, XX, t(4;7)(q35;q22), t(9;13)(p22;q22), 46, XX, add(1)(p36.1), t(4;7)(q35;q22), t(9;13)(p22;q22). Because KY-3 cell line consisted of 2 different clones, we used KY-1 and KY-2 for further analysis. Because Ig heavy chain rearrangement was negative in the cell lines both by PCR and Southern blot analysis, we assessed the expression of genes serially expressed in B lymphoid lineages, such as Pax5, PRDM1, Xbp1, to confirm the origin of the tumor. Reverse transcription-PCR showed the expression of both spliced and unspliced forms of Xbp1 [Xbp1(s) and Xbp1(u), respectively], but the expression of Pax5 or PRDM1 was not detected in the established cell lines (Fig. 2D), suggesting that the expression of Xbp1(s) mRNA contributes to the plasmacytic appearance in the cell lines.

Electron microscopy was used for the analysis of ultrastructure of the cell lines. Contrary to the expression of plasmacytic markers such as CD138, EMA, and VS38c, the cell lines did not show abundant endoplasmic reticulum (ER) formation, which is a hallmark of mature plasma cells (Fig. 2E; 18).

**CGH and FISH analysis**

We performed CGH analysis using KY-2 cell line harboring t(4;7) and t(9;13) to identify the chromosome break points. Copy number alteration was identified at around 182,000,000 [based on National Center for Biotechnology Information (NCBI) Build 36.3 database] on chromosome 4. No significant difference in the copy number was seen on other chromosome regions. FISH analyses using a series of...
Identification of Genetic Events in Plasmablastic Lymphoma

BAC probes to narrow down the candidate regions containing the chromosome break points was subsequently performed, which led to the identification of translocation within BAC clone 1095A04 on chromosome 4, 0876E06 on chromosome 7, 1021D11 on chromosome 9, and between 0367C11 and 1135C06 on chromosome 13, respectively (Fig. 3A). Computer-based search of genes located on these regions from the NCBI database was performed, and altogether 4 genes [methylthioadenosine phosphorylase (MTAP), cyclin-dependent kinase (CDK) inhibitor 2A (CDKN2A), 2B (CDKN2B), ATP-binding cassette, subfamily B (MDR/TAP), member 1 (ABCB1/MDR-1)] were identified as candidate genes involved in the current translocation (Fig. 3B and Supplementary Fig. S1).

**t(9;13)**

Three genes (MTAP, CDK inhibitor 2A and 2B) located on chromosome 9 were identified as possible candidates...
involved in the chromosomal translocation (Fig. 3B and Supplementary Fig. S1). Because CDK inhibitors are related to the pathogenesis of various cancers, we focused on analyzing the expression of CDK inhibitor 2A and 2B. **CDKN2A** includes 2 splice variants, isoform 1 (*p16*) and isoform 4 (*p14*). **CDKN2B** encodes tumor suppressor *p15*. Although mRNA of all *p14*, *p15*, and *p16* was detected by reverse transcription-PCR in KY-1 and KY-2 (Fig. 4A), protein expressions were only positive for *p15* (Fig. 4B). In order to identify the mechanism underlying positive mRNA expression and loss of protein expression within the tumor cells, we analyzed the mRNA sequence of the coding region for *p16* mRNA. The result showed a nonsense mutation of C to G in the third nucleotide of codon 129, resulting in a premature stop at codon number 129.

No genes were identified on chromosome 4 within the range of the BAC clone 1095A04. **MDR-1** was located on chromosome 7, and thus was considered to be upregulated in cell lines harboring this translocation (KY-2; Supplementary Fig. S1). Flow cytometry was performed on KY-1 and KY-2. CD138-positive KY-1 cells, which do not carry t(9;13), did not express MDR-1 on the cell surface, whereas CD138-positive KY-2 cells expressed MDR-1 (Fig. 5A). The function of the MDR-1 protein was confirmed by dye efflux assay. KY-2 cell line expressing MDR-1 efficiently effluxed rhodamine 123 dye after 1 hour incubation (Fig. 5B, right). In contrast, KY-1 cells without MDR-1 retained rhodamine 123 dye (Fig. 5B, left). These results indicated that MDR-1 protein is working as an efflux pump in KY-2 cell line.

**In vitro drug sensitivity test**

To assess whether the upregulation of MDR-1 contributed to increased resistance to chemotherapeutic agents, and to evaluate whether our current cell line could be used to assess drug sensitivity *in vitro*, proliferation assay was performed using the 2 cell lines, KY-1 and KY-2. Both KY-1 and KY-2 showed reduced proliferation against CDDP, DXR, and bortezomib in a dose-dependent manner. KY-2 that express MDR-1 showed increased resistance compared to KY-1 in terms of CDDP and DXR treatment (Fig. 6). Therefore, we confirmed that the expression of MDR-1 played a role in the acquisition of drug resistance in the current tumor, and we have also assessed the efficacy of a different chemotherapeutic agent, bortezomib, as a candidate drug for the treatment of this type of tumor.
Discussion

We have experienced a rare case of plasmablastic lymphoma in an otherwise healthy Japanese woman and established cell lines from the original tumor. To our knowledge, this is the first report in the literature of the establishment of cell line for this disease.

Two novel chromosomal translocations were identified in the current patient sample; t(4;7)(q35;q22) and t(9;13) (p22;q22). Since neither translocations were evident in the bone marrow mononuclear cells of the patient, these translocations were considered to be tumor specific. Chromosomal breakpoint was identified using conventional FISH analysis in combination with CGH array. Array CGH has been used as a useful tool for discovering copy number changes, to identify small deletions or amplifications at the breakpoints and to understand the genetic background of various cancers (21–23). Chang and colleagues (24) have recently reported on their successful finding that compared genomic profiling of plasmablastic lymphoma and DLBCL, plasmacytic myeloma using array CGH technique. In our case, we were only able to identify 1 region of the 4 possible breakpoints by array CGH method. One reason might be due to the fact that we used commercially available pooled DNA from anonymous donors as the control since we could not obtain DNA from nontumor tissue of the patient, and thus the change in copy number was masked among the difference between individuals.

Moreover, the chromosomal translocation may not have caused any detectable copy number change in the first place. However, we were able to identify 1 locus on chromosome 4 that matched the result of the conventional FISH analysis. Together with previous reports, we consider CGH analysis to be a useful technique for identifying chromosomal breakpoint in future cases.

Breakpoint analysis of the current patient’s tumor identified 2 genes possibly involved in the translocation. One was tumor suppressor gene p16 and the other MDR-1. Considering the clinical course of the patient, that she gradually lost response to chemotherapy, and the karyotype of the patient’s sample, we assume that loss of p16 expression at the protein level led to the susceptibility to oncogenic events in this tumor, and the upregulation of MDR-1 to treatment refractoriness. Although not specific for plasmablastic lymphoma, p16 is known to be down regulated in various lymphoid malignancies due either to deletions, mutations, or promoter methylation, and has been proposed to be related to poor prognosis in some large clinical trials (19, 20, 25–27). 9p21 deletion is known to be a relatively frequent event in pediatric acute lymphoblastic leukemia (ALL), and in cases with heterozygous deletions, the remaining alleles were found to be hypermethylated, leading to loss of p16 expression (28, 29). The loss of p16 protein expression and its causal relationship with the translocation t(9;13) in our cell line is further to be determined; however, we hypothesized that the translocation t(9;13) has led to the disruption of p16 gene in 1 allele and a concomitant mutation in the other allele has led to the loss of protein expression. The identification of a nonsense mutation at codon 129 by sequence analysis partly proved this hypothesis. Villuendas and colleagues (30) have previously identified the same mutation in a Burkitt lymphoma cell line, supporting the fact that this mutation has led to the loss of p16 protein expression and to lymphomagenesis in the current patient’s tumor.

MDR-1 upregulation is a well-known mechanism for acquiring drug resistance in many tumors. Mickley and colleagues (31) have reported on the upregulation of MDR-1 after a 4;7 chromosomal translocation in a cultured colon adenocarcinoma cell line after a prolonged exposure to chemotherapeutic agents. A couple of other reports have stated on other chromosomal translocations leading to the expression of MDR-1 protein (32, 33). Knutsen and colleagues (34) have found 2 patients with rearrangement of MDR-1 gene leading to refractory ALL. Chromosomal translocation together with stabilization of mRNA and translational initiation of MDR-1 is considered to be one of the major causes of acquiring drug resistance in hematologic malignancies (35). The translocation t(4;7)(q35; q22) is a novel translocation leading to the upregulation of MDR-1, and identification of the precise mechanism leading to its expression is left for further investigation.

One other unique feature of our case is that the immunoglobulin heavy chain rearrangement was negative both in the patient sample and the established cell line. Thus, in order to identify the origin of the tumor, we assessed genetic events involved in the development of B lymphocytes. Pax5 and PRDM1 were negative, but both the unspliced and spliced forms of Xbp1 were positive in our cell line. Maestre and colleagues (36) have recently assessed the expression of Xbp1(s) to be highly associated with plasmablastic feature among diffuse B cell lymphomas. Therefore, we believe that some genetic alteration leading to the expression of Xbp1(s) leads to the plasmablastic feature of the current tumor.
Considering the plasmablastic feature of the tumor, we assessed the effect of bortezomib, a proteasome inhibitor, on the cell line. Bortezomib has been reported to show its effect on myeloma cells through the inhibition of Xbp1s accumulation (37). Bortezomib was effective in our tumor cell line in vitro regardless of MDR-1 expression. This finding is consistent with the report from Lu and colleagues (38) showing that bortezomib is effective in MDR-1 expressing leukemia cell line, K562/A02. It also supports the report by Bose and colleagues (39) where bortezomib showed marked shrinkage of the disease in a patient with HIV-positive plasmablastic lymphoma. Therefore, we believe that the use of bortezomib with or without conventional chemotherapeutic agents may be an effective treatment strategy for plasmablastic lymphomas.

Although we have extensively analyzed the genetic events from 2 unique chromosomal translocations in this patient with plasmablastic lymphoma, the limitations of our current work is that the finding was only confirmed in this single case. Since plasmablastic lymphoma is a rare subtype of non-Hodgkin’s lymphoma, and currently there is no other cell line available for this disease, we were unable to identify the same genetic events in other samples. Therefore, the universality of the currently identified genetic events needs to be further elucidated by increasing the number of samples.

In summary, we have established a novel cell line of plasmablastic lymphoma carrying 2 novel chromosomal translocations unique to the patient. Evaluation of the genes involved in the translocation identified loss of p16 expression at the protein level and upregulation of MDR-1 as the possible causes involved in this event. Sensitivity to chemotherapeutic agents could be assessed using the currently established cell lines.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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