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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**Running Title:** Identification of genetic events in plasmablastic lymphoma

**Key Words:** plasmablastic lymphoma, p16, MDR-1, Xbp1
Statement of Translational Relevance

This report is to our knowledge the first report that has established a cell line of plasmablastic lymphoma, and assessed the genetic events from unique chromosomal translocations involved in the pathogenesis of the disease. The translocation led to the loss of p16 expression at the protein level as one of the initiating events for the oncogenesis of this lymphoma, and upregulation of MDR-1 protein as the acquisition of resistance to chemotherapeutic agents. Expression of Xbp1(s), one of the key transcription factors for plasmacytic differentiation, has also been identified as a related event to the plasmablastic appearance of the tumor. In vitro drug sensitivity test was performed using this cell line, which showed resistance to cisplatin and doxorubicin, however showed sensitivity to bortezomib. This cell line will improve our understanding of the pathogenesis of plasmablastic lymphoma, and also serve as a good tool to develop innovative therapies for this disease.
Abstract

Purpose: To establish cell lines from the patient with plasmablastic lymphoma, who was immunologically competent including negative 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 were attempted by inoculating the patient’s lymph node biopsy specimen subcutaneously to immunodeficient mice. 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, RT-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 Pax5 or PRDM1, but was positive for 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 et al. (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 cases die within 1 year of the onset, 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 due to the lack of disease model, cell lines and disease-specific chromosome or gene aberrations.

We newly established three cell lines from the lymph node specimen of the patient with plasmablastic lymphoma, each harboring unique chromosomal aberration seen in the patient’s tumor, and analyzed genetic events involved in these translocations. To our knowledge, this is the first report of the establishment of a cell line of plasmablastic lymphoma, in which a concise genetic evaluation has been performed.

Materials and Methods

Patient

A 47-year-old female presented to our hospital for irregular menstrual cycle, with massive menorrhagia following two months of amenorrhea. Transvaginal ultrasound and computed tomography (CT), magnetic resonance imaging (MRI) showed a mass of 48 x 35 x 32 mm in the uterus, protruding into the abdominal cavity, the enlargement of bilateral ovary, supraclavicular lymph node and multiple peritoneal masses (Figure 1A and data not shown). Emergent hysterectomy, bilateral adnexectomy, omentectomy and lymph node sampling was performed, but she developed supraclavicular and axillary
lymph node swelling approximately one week after the operation, together with high fever. Under the tentative diagnosis of endometrioid carcinoma of the uterus, she was treated with cisplatin (CDDP) 50 mg/m² (day 1) and doxorubicin (DXR) 60 mg/m² (day 1; AP regimen), and marked shrinking of the lymph nodes was obtained.

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 (Figure 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 to 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 (NOG) mice(13) and subsequently to nude mice. The lymph node was simultaneously dispersed and cultured in RPMI1640 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FBS. The subcutaneous tumor from NOG 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., Tokyo, Japan). Immunophenotyping using flow cytometry was performed at the clinical laboratory of our hospital. Ultrastructure of the cell lines was analyzed by electron microcopy as previously described.(14)
Comparative genomic hybridization analysis

Comparative genomic hybridization (CGH) array made by NimbleGen (currently Roche NimbleGen, Madison, WI, USA) that covers the entire regions of chromosome regions 4q35, 7q22, 9p22, 13q22 with 5-Mbp margin on both ends was used. The probes were 50-75 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, Hilden, Germany). Human genomic DNA from multiple anonymous donors (female) (Promega Corporation, Madison, WI, USA) was used as control template. Analysis of CGH array was performed by Roche Diagnostics KK (Tokyo, Japan).

Fluorescence in situ hybridization analysis

BAC clones corresponding to the regions 4q35, 7q22, 9p22, 13q22 were selected from Keio BAC library(15) every 3 Mbps and subsequently narrowed down (Supplementary Table 1). 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 (San Diego, CA, USA). Mouse anti-p14 antibody (14P01) was a product of Thermo Fisher Scientific (Fremont, CA, USA) and both rabbit anti-p15 antibody (sc-612) and rabbit anti-p16 antibody (sc-468) were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Rabbit anti-β-actin antibody was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

**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, Brea, CA, USA). 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. HRP-conjugated sheep anti-mouse IgG antibody or HRP-conjugated
donkey anti-rabbit IgG antibody (GE Healthcare, Buckinghamshire, UK) were used as secondary antibodies. The proteins were detected using ECL Western Blotting Detection Reagents and Hyper ECL film (GE Healthcare).

**RT-PCR**

Total RNA was extracted using RNeasy Mini Extraction Kit (Qiagen). 0.5 µg of total RNA was subjected to reverse transcription using SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA) and PCR as per manufacturer’s protocol. Expression of the target genes were assessed using primer pairs described in supplementary table 2. Takara Ex Taq Hot Start version (Takara Bio Inc, Shiga, Japan) was used for PCR amplification. HeLa cells were used as positive control for p14, p15 and p16 analysis. RPMI8226, Daudi, U266 cells were used as a positive control for Xbp1, Pax5 and PRDM1 analysis, respectively.

**Sequence Analysis**

RT-PCR and PCR amplification of the coding region of p16 mRNA was performed as previously described using the primer as noted in supplementary table 2. The product was sequenced using BigDye terminator cyclesequencing kit (Applied Biosystems,
Carlsbad, CA, USA) 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/200μl in 96-well plates with various concentrations of cisplatin, doxorubicin or bortezomib. Ten μl of WST-8 reagent (Kishida Chemical, Osaka, Japan) was added to each well during the last 4 hour of culture. Absorbance was measured at a wavelength of 450 nm using 96-well microplate reader, Model 680 (Bio-Rad, Hercules, CA, USA). Each experiment was performed in triplicate and three 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**

*Establishment and characterization of cell lines*

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 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 (Figure 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, one from the original patient tumor from the lymph node and two 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 (Figure 2B). Immunophenotype of all three cell lines were 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 (Figure 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). Since KY-3 cell line consisted of two different clones, we used KY-1 and KY-2 for further analysis.

Since 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, paired box 5 (Pax5), PR domain containing 1, with ZNF domain (PRDM1), X-box binding protein 1 (Xbp1), to confirm the origin of the tumor. RT-PCR showed the expression of both spliced and unspliced form of Xbp1 (Xbp1(s) and Xbp1(u), respectively), but the expression of Pax5 or PRDM1 was not detected in the established cell lines (Figure 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 (Figure 2E).
**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 BAC probes to narrow down the candidate regions containing the chromosome break points was subsequently performed, that 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 (Figure 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, sub-family B (MDR/TAP), member 1 (ABCB1/MDR-1)) were identified as candidate genes involved in the current translocation (Figure 3B and Supplementary Figure 1).

\[ t(9;13) \]

Three genes (MTAP, CDK inhibitor 2A and 2B) located on chromosome 9 were
identified as possible candidates involved in the chromosomal translocation (Figure 3B and Supplementary Figure 1). Since CDK inhibitors are related to the pathogenesis of various cancers, we focused on analyzing the expression of CDK inhibitor 2A and 2B. CDKN2A includes two 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 RT-PCR in KY-1 and KY-2 (Figure 4A), protein expression was only positive for p15 (Figure 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 (Figure 4C). Although the causal relationship between the chromosomal translocations and the loss of both p14 and p16 protein expression needs to be further elucidated, since it has been known that the loss of tumor suppressor p16 plays a key role in leukemogenesis and lymphomagenesis(19-20), we consider that the downregulation of p16 protein may account for the susceptibility to tumorigenesis in this plasmablastic lymphoma.

\[ t(4;7) \]

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 Figure 1).

Flow cytometry was performed on KY-1 and KY-2. CD138-positive KY-1 cells, which does not carry t(9;13), did not express MDR-1 on the cell surface, whereas CD138-positive KY-2 cells expressed MDR-1 (Figure 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 (Figure 5B, right panel). In contrast, KY-1 cells without MDR-1 retained rhodamine 123 dye (Figure 5B, left panel). 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 two 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 (Figure 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 CC et al.(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 one 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 non-tumor 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 one 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 breakpoints in future cases.

Breakpoint analysis of the current patient’s tumor identified two 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)
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 one 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 et al.\(^{(30)}\) 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 et al.\(^{(31)}\) 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 et al.\(^{(34)}\) 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. 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 form of Xbp1 was positive in our cell line. Maestre et al. 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 Xbp1(s) accumulation. Bortezomib was effective in our tumor cell line in vitro regardless of MDR-1 expression.
This finding is consistent with the report from Lu et al.(38) showing that bortezomib is effective in MDR-1 expressing leukemia cell line, K562/A02. It also supports the report by Bose et al.(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 two 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 two 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.

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Figure Legends

Figure 1. Imaging and pathological study of the patient with plasmablastic lymphoma.

A) MRI scan of the patient before surgery showed a large tumor in the uterus. B) Pathological findings of the resected uterus tumor. Hematoxylin and eosin (H&E) staining showed proliferation of large cells with abundant cytoplasm resembling the features of plasma cells. Those cells were positive for CD138, EMA and VS38c.

Figure 2. Analysis of the newly established plasmablastic lymphoma cell lines, KY-1 and KY-2. A) Pathology specimen of the tumor formed in the subcutaneous tissue of NOG mice. Histological findings were identical to the original tumor shown in Figure 1B. Plasmacytic markers of CD138, VS38c and EMA were all positive. B) Giemsa staining of KY-2 cells. The cells had prominent nucleoli and eosinophilic cytosol, suggestive of plasmacytoid appearance. C) 92.3% of KY-2 cells were CD138+CD16- by flow cytometry. D) Pax5 and PRDM1 expression was not seen in either KY-1 or KY-2. However, both Xbp1(u) and Xbp1(s) was positive by RT-PCR. Expression of β-actin was used as a control. Daudi, U266 and RPMI8226 cells were used as a positive control for Pax5, PRDM1 and Xbp1 expression, respectively. Negative control consisted of water. E) Ultrastructure of KY-2 cells by electron microscopy. Formation of endoplasmic reticulum
was not enriched in the cells, consistent with the negative immunoglobulin excretion of the cells.

**Figure 3.** Mapping of the chromosomal breakpoints for t(4:7) and t(9:13) using FISH analysis. A) KY-2 cells were fixed in metaphase and stained with BAC probes. Arrows indicate positive signal on the untranslocated chromosomes. Arrow heads indicate positive signal on the translocated chromosomes. Each picture shows additional signal on the translocated chromosome, indicating that the translocation occurred between the two probes. B) Schematic diagram of the results of the translocation.

**Figure 4.** Loss of p16 tumor suppressor protein in the established cell lines. A) mRNA was detected by RT-PCR analysis for all of p14, p15 and p16 in both KY-1 and KY-2 cells. HeLa cells were used as positive control. No template was added in the negative control. B) Both p14 and p16 protein expression was not detected in either cell lines by Western blot analysis. In contrast, p15 was detectable in both KY-1 and KY-2 cells. HeLa cells were used as positive control. C) Sequence analysis of the coding regions of p16 mRNA revealed a substitution of cytosine to guanine at nucleotide number 389 leading to an immature stop at codon number 129.
Figure 5. Expression and function of MDR-1. A) CD138-positive cells in KY-2, that harbors two translocations, t(4;7) and t(9;13), were positive for MDR-1 expression. In contrast, CD138-positive cells in KY-1, that carries only t(9;13), was negative for MDR-1 expression. These results indicate that the additional translocation of t(4;7) may be the cause of MDR-1 expression in KY-2 cells. B) KY-1 cells retained rhodamine 123 dye after 1 hour incubation. MDR-1 expressing KY-2 cells excreted the dye, showing that the MDR-1 protein in KY-2 cells was functional.

Figure 6. Proliferation assay using KY-1 and KY-2 cells. KY-1 was more sensitive to various drugs compared to KY-2, that expresses MDR-1, except for bortezomib.

* indicates p<0.05 for comparison between KY-1 and KY-2. Data shown are expressed as average +/- S.D. of three experiments. CDDP: cisplatin, DXR: doxorubicin.
Figure 1
Figure 2

A. H&E stained section showing CD138 and EMA.

B. CD138 and VS38c stained section.

C. Flow cytometry analysis of KY-2 cells showing positive expression of Pax5, PRDM1, Xbp1 (u), and Xbp1 (s) compared to KY-1 cells.

D. Western blot analysis of KY-2 cells showing positive expression of Xbp1 (u) and Xbp1 (s) compared to KY-1 cells.

E. Electron microscopy image of KY-2 cells.
Figure 3
B

Chromosome No. 4
Chromosome No. 7

1095A04

0876E06

ABCB1/MDR-1

Chromosome No. 9
Chromosome No. 13

p21.3
q31.1

0367C11

1021D11

CDKN2B
CDKN2A

MTAP

Figure 3
Figure 5
Figure 6
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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