Genomic rearrangements involving programmed death ligands are recurrent in primary mediastinal large B-cell lymphoma

Short title:

PDL rearrangements in PMBCL

Authors:

David D. W. Twa,¹,² Fong Chun Chan,¹,³ Susana Ben-Neriah,¹ Bruce W. Woolcock,¹ Anja Mottok,¹ King L. Tan,¹ Graham W. Slack,¹,² Jay Gunawardana,¹,² Raymond S. Lim,¹ Andrew W. McPherson,³ Robert Kridel,¹,² Adele Telenius,¹ David W. Scott,¹ Kerry J. Savage,¹,² Sohrab P. Shah,² Randy D. Gascoyne,¹,² and Christian Steidl¹,²

Affiliations:

¹Centre for Lymphoid Cancer, BC Cancer Agency, Vancouver, BC; ²Department of Pathology and Laboratory Medicine, University of British Columbia, Vancouver, BC; and ³Bioinformatics Training Program, University of British Columbia, Vancouver, BC

Corresponding author:

Christian Steidl, Department of Experimental Therapeutics, BC Cancer Research Centre, 675 West 10th Avenue, Vancouver, BC, Canada, V5Z 1L3; e-mail: csteidl@bccancer.bc.ca
Key Point
Programmed death ligands 1 and 2 are rearranged at a frequency of 20% in primary mediastinal large B-cell lymphoma.

Abstract
The pathogenesis of primary mediastinal large B-cell lymphoma (PMBCL) is incompletely understood. Recently, specific genotypic and phenotypic features have been linked to tumor cell immune escape mechanisms in PMBCL. We studied 571 B-cell lymphomas with a focus on PMBCL. Using fluorescence in situ hybridization, here, we report that the programmed death ligand (PDL) locus (9p24.1) is frequently and specifically rearranged in PMBCL (20%) as compared to diffuse large B-cell lymphoma, follicular lymphoma and Hodgkin lymphoma. Rearrangement was significantly correlated with over-expression of PDL transcripts. Employing high-throughput sequencing techniques, we characterized novel translocations and chimeric fusion transcripts involving PDLs at base-pair resolution. Our data suggest that recurrent genomic rearrangement events underlie an immune privilege phenotype in a subset of B-cell lymphomas.

Introduction
Primary mediastinal large B-cell lymphoma (PMBCL) is an aggressive disease known to share certain genotypic and phenotypic features with classic Hodgkin lymphoma (CHL) and diffuse large B-cell lymphoma (DLBCL). However, the complete landscape of genetic alterations involved in PMBCL pathogenesis has yet to be fully elucidated.
Among the most common chromosomal alterations in PMBCL are amplifications of chromosome 9p and translocations involving \textit{CIITA} (16p13.13).\textsuperscript{4-7} These aberrations have been suggested to affect tumor-microenvironment interactions resulting in immune privilege.\textsuperscript{7,8} Here, we demonstrate that rearrangements involving immune cell anergy-inducing programmed death ligand (PDL) 1 (\textit{CD274}) and 2 (\textit{PDCD1LG2}) are recurrent in and characteristic of PMBCL. Further, we show such rearrangements are correlated with elevated transcript levels and we characterize novel translocations identified using high-throughput sequencing.

\textbf{Methods}

We studied 571 primary B-cell lymphoma samples in conjunction with 17 established B-cell-derived cell lines. Using in-house bacterial artificial chromosome probes, fluorescence \textit{in situ} hybridization (FISH) or FISH combined with CD30 immunofluorescence (in the case of CHL specimens) was performed to characterize the PDL locus.\textsuperscript{7,9,10} These cases were also analyzed with Epstein-Barr virus-encoded RNA \textit{in situ} hybridization (EBER-ISH). Quantitative real-time PCR (qRT-PCR) was performed for \textit{CD274} and \textit{PDCD1LG2} transcript expression on a subset of cases from the FISH cohort (N=76). Surface \textit{PDCD1LG2} expression of the cell lines was determined via flow cytometry. To characterize the Hodgkin cell lines found to be rearranged by FISH, one whole-transcriptome (RNA-seq) library (CHL-derived L-428) was reanalyzed and one new whole-genome library (L-428) and two new RNA-seq libraries (CHL-derived L-1236 and nodular lymphocyte predominant Hodgkin-derived DEV) were sequenced.\textsuperscript{7} This
study was approved by the BC Cancer Agency (REB: H11-00684) and conducted in accordance with the Declaration of Helsinki.

**Results and discussion**

There is increasing evidence that genes involved in tumor-microenvironment interactions play a crucial role in the pathogenesis of B-cell lymphomas.\(^8\) Two of such genes, *CD274* and *PDCD1LG2*, have been implicated in promoting tumor cell immune evasion.\(^{11,12}\) These ligands have been reported to be over-expressed in solid tumors, including lymphomas, but the mechanisms by which over-expression occurs are incompletely understood.\(^{4,7,13-15}\) Using an in-house break-apart FISH assay (Figure S1, Table S1), we determined the frequency of PDL locus aberration in 17 lymphoma-derived cell lines (Table S2) and 571 clinical lymphoma samples (Figure 1). This analysis revealed a break-apart frequency of 20% and an amplification frequency of 29% in 125 PMBCL cases. The prevalence of PMBCL break-apart events was significantly higher than in DLBCL, primary central nervous lymphoma (PCNSL), primary testicular DLBCL (TDLBCL) and follicular lymphoma (FL) \((P < 0.05)\). No significant differences of any noted clinical parameters, including treatment outcome variables (progression-free and overall survival), were observed between PDL rearranged, amplified and non-rearranged cases.

To study the correlation of genomic rearrangements and copy-number changes of the PDL locus with gene expression, we performed qRT-PCR on 17 cell lines and 76 clinical cases that had fresh frozen material available (Table S3). These cases were stratified according to the genomic aberration status as determined by FISH. In break-
apart positive cases across all lymphomas, \textit{PDCD1LG2} transcript levels were significantly higher as compared to neutral (\(P=0.0003\)), gained (\(P=0.001\)) and amplified (\(P=0.005\)) loci (Figure 1; Table S4). A significant difference was also observed between \textit{CD274} transcript levels of rearranged and copy-neutral cases (\(P=0.03\)), though only a trend was found when compared to copy-number aberrated cases. This reduced level of correlation may be attributable to the heterogeneity in rearrangement anatomy which disparately affects the PDL loci. The observation that \textit{CD274} and \textit{PDCD1LG2} expression levels were also elevated (relative to PDL expression in reactive tonsil cells) in cases where no rearrangement could be observed via FISH suggests alternative mechanisms of deregulation exist. These mechanisms might include epigenetic and microRNA regulatory factors.\textsuperscript{16,17} As Epstein-Barr virus (EBV) infection has been implicated in PDL expression, we performed EBER-ISH (Table S1).\textsuperscript{18} No significant correlation was observed between EBV positivity and either PDL-break-apart or transcript levels, although the small number of EBV positive cases in our study limits statistical power.

To determine the rearrangement structures which were observed at the PDL locus, we reanalyzed previously published RNA-seq data of L-428.\textsuperscript{7} Additionally, we sequenced the whole genome of L-428 and the whole transcriptomes of L-1236 and DEV. Using genomic breakpoint and fusion transcript predicting algorithms, we mined these data with a specific focus on the 9p24.1 locus (Tables S5 and S6). We identified three novel fusion transcripts within this locus that were validated by Sanger sequencing: \textit{CIITA-PDCD1LG2} (DEV), \textit{KIAA1432-CLDN14} (L-428) and \textit{PDCD1LG2-IGHV7-81} (L-1236) (Figure 2). The fusion transcript observed in DEV was found to be
similar to two fusions we have previously reported in clinical PMBCL cases. While clearly involving the 9p24.1 locus, the FISH break-apart signal pattern of L-428 was attributed to a translocation 60 kb downstream of \textit{PDCD1LG2}. The mechanism by which this translocation increases PDL expression is unknown; though \textit{KIAA1432-JAK2} fusions have recently been implicated in small cell lung cancer. The chimeric transcript produced by L-1236 was predicted to generate a C-terminal-truncated \textit{PDCD1LG2} protein. In this instance, the receptor binding site, coded in the fourth exon, was left intact.

To investigate the effect of these rearrangements on protein expression, we performed flow cytometry on several cell lines with a specific focus on those found harbouring 9p24.1 translocations: L-428, L-1236 and DEV. We found elevated expression of \textit{PDCD1LG2} in all three rearranged cell lines compared to U-HO1 and DOHH-2 (normal loci) (Figure S2). Further, expression levels in L-428 and L-1236 were determined to be in agreement with the literature. \textit{PDCD1LG2} protein surface levels were highest in DEV and were also appreciably greater than in cell line MEDB-1 (amplified locus). This shows that a \textit{PDCD1LG2} epitope is readily detectable in DEV cells at elevated levels corresponding to a \textit{CIITA-PDCD1LG2} fusion that might alter T-cell activity states in the microenvironment as previously demonstrated.

By merging these data of this study with the three PDL fusions previously reported by our group (Figure 2), we have validated the presence of 5' fusion transcripts involving PDL in immortalized cells and have established the existence of a novel fusion transcript which involves the 3’ end of \textit{PDCD1LG2}. As substantially elevated transcript levels were observed in all instances with validated direct involvement of PDL
rearrangements, up-regulated expression of PDLs may be a consistent mechanism that contributes to pathogenesis. Deregulated transcript expression is believed to arise via juxtaposing either a highly active promoter (CIITA) or enhancing elements (IGHV7-81) adjacent to PDLs. Other potential selective advantages these translocations confer might be related to the altered function of the involved fusion partner genes. This would include decreased tumor immunogenicity as a result of reduced MHC II expression from CIITA-associated translocations. Another potential benefit specific to the 3' translocation in L-1236 is the loss of the transcript 3' UTR. This might lead to a loss of microRNA binding sites increasing the half-life of PDL transcript and/or the loss of the exons coding for the transmembrane domain region which may act to solubilize the protein.

Taken together, our findings establish that rearrangement of the PDL locus is recurrently selected for and that such rearrangements lead to elevated transcript levels and/or the production of chimeric PDL fusion transcripts. Recently, phase I/II clinical trials targeting the PDL pathway in solid tumors have shown promising outcomes. Our data strongly suggest that PDLs and their high-affinity receptor, programmed death 1 (PDCD1), are rational drug targets in a subgroup of B-cell lymphomas characterized by PDL locus aberrations.
Acknowledgements

Operational funds for this work were available through the Canadian Institutes of Health Research Grant #111043 to R.D.G. and the Terry Fox Research Institute (Terry Fox New Frontiers Program in Cancer, #1023) to C.S. This work was also generously supported by the British Columbia Cancer Foundation. C.S. and S.P.S. are recipients of Career Investigator Awards from the Michael Smith Foundation for Health Research (MSFHR). A.M. is supported by a Mildred-Scheel-Cancer-Foundation Fellowship. D.D.W.T is supported by UBC fellowships and the Canadian Hematology Society.

Authorship Contribution: D.D.W.T. designed and performed the research, interpreted the data and wrote the manuscript. F.C.C., R.S.L. and A.W.M. analyzed sequencing data and contributed to the figures. S.B.N., B.W.W., K.L.T., A.M. and G.W.S. produced and analyzed data. J.G., R.K., A.T. assisted with data collection. D.W.S., K.J.S. and S.P.S. provided editorial input. R.D.G. analyzed data, constructed the database and edited the paper. C.S. designed the research, analyzed data, wrote the manuscript and approved the paper.

Conflict-of-interest disclosure: The authors declare no competing interests.

Correspondence: Christian Steidl, Department of Experimental Therapeutics, BC Cancer Research Centre, 675 West 10th Avenue, Vancouver, BC, Canada, V5Z 1L3; e-mail: csteidl@bccancer.bc.ca
References


Figure 1. Frequency of PDL locus alterations and correlation with transcript expression levels. (A) Representative FISH signal patterns from two clinical samples; top: break-apart (primary PMBCL), bottom: amplification (primary PMBCL). (B) The proportion of PDL locus (9p24.1) aberration across seven different subtypes of B-cell lymphomas observed using an in-house break-apart FISH assay (N values: DLBCL=134, PCNSL=130, TDLBCL=82, PMBCL=125, CHL=20, NLPHEL=12, FL=68). P values were <0.05 for the number of break-apart cases in PMBCL in comparison to lymphomas with N>12; P<0.05 for the number of amplified cases in PMBCL in comparison to lymphomas with N>20. (C) CD274 transcript expression in 17 cell lines and 76 clinical samples as determined via qRT-PCR (N values: PMBCL=48, DLBCL=19, TDLBCL=6, PCNSL=3). The dotted red line represents basal CD274 expression as determined by reactive tonsil cells. The break-apart to neutral comparison reached statistical significance (P=0.03). Amplified to neutral (P=0.001) and amplified to gain (P=0.01) comparisons also reached statistical significance. (D) PDCD1LG2 transcript expression in the cohort described above. The dotted blue line represents basal PDCD1LG2 expression. Statistical significance was researched between break-apart to neutral (P=0.0003), break-apart to gain (P=0.001) and break-apart to amplified (P=0.005) case comparisons. The amplified to neutral comparison also reached statistical significance (P=0.002). (E) Differential expression of both PDL transcripts in break-apart cases. Dots circled in red (two) were Sanger validated to have a translocation involving the CD274 locus while those in blue (five) were found to harbour rearrangements of PDCD1LG2. Note how break-apart events result in disparate expression levels between transcripts.
Figure 2. A summary of known translocations involving the 9p24.1 locus in PMBCL and two novel fusion transcripts. (A) Translocations involving CD274 are depicted in grey while those that are involved or situated downstream from PDCD1LG2 are depicted in black. Dot size and adjoining line thickness qualitatively depict the number of cases involving those loci; the CIITA-PDCD1LG2 line represents four cases. Three of the six depicted translocations are novel, PDCD1LG2-IGHV7-81, CIITA-PDCD1LG2, and KIAA1432-CLDN14. (B) Sanger validated fusion transcripts involving PDCD1LG2; all seven exons of PDCD1LG2 are depicted in dark grey and are in scale to one another. Known and suspected protein coding regions are drawn with a greater exon width.
Genomic rearrangements involving programmed death ligands are recurrent in primary mediastinal large B-cell lymphoma