Differential and tumor-specific expression of CD160 in B-cell malignancies

Short Title: Tumor-specific expression of CD160

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Abstract

CD160 is a human NK-cell activating receptor, also expressed on T-cell subsets. Here we examined 811 consecutive cases of B-cell lymphoproliferative disorders (B-LPD), and demonstrate CD160 expression in 98% (590/600) of CLL cases, 100% (32/32) of hairy cell leukemia (HCL), 15% (5/34) of mantle cell lymphoma (MCL) in leukemic phase and 16% (23/145) of other B-LPD. CD160 transcript and protein were absent in the normal B-cell hierarchy, from stem cells, B-cell precursors, maturing B-cells in the germinal center and circulating B-cells, including CD5⁺CD19⁺ B1-cells in umbilical cord. CD160 positivity was significantly higher in CLL and HCL in terms of percentage (65.9 and 67.8%, respectively, \( P < 0.0001 \)) and median fluorescence intensity (552 and 857, respectively, \( P < 0.0001 \)) compared to all other B-LPD. Lymph node CLL samples were also CD160⁺. Using the disease-specific expression of CD5, CD23 and CD160, a score of 3 characterized CLL (diagnostic odds ratio 1430); a score of 0 excluded CLL, MCL and HCL; and the CD23/CD5 ratio differentiated CLL from leukemic CD23⁺ MCL. In the B-cell lineage, CD160 is a tumor-specific antigen - known to mediate cellular activation signals in CLL - and a novel target for therapeutic manipulation and minimal residual disease monitoring.

Keywords

CD160, CLL, mature B-cell lymphoid leukemias, flow cytometry, tumor antigen
Introduction

CD160 is an immunoglobulin (Ig)-like activating natural killer (NK) cell receptor expressed on the majority of circulating NK cells and on a subset of circulating cytotoxic T-cells, but not B-cells or EBV-transformed B-cell lines.\textsuperscript{1,2} In contrast to the majority of NK cell receptor genes located on chromosomes 12 and 19,\textsuperscript{3} the \textit{CD160} gene is located on chromosome 1q42.3\textsuperscript{4} CD160 is expressed by most peripheral blood TCR\textgamma\textdelta lymphocytes, a minor subset of circulating CD8\textsuperscript{bright+} TCR\textalpha\textbeta cells and all small intestinal intraepithelial T lymphocytes, phenotyped as CD3\textsuperscript{+}TCR\textalpha\textbeta CD56\textsuperscript{neg}.\textsuperscript{5} A minor population of CD4+ T-cells also express CD160.\textsuperscript{6} CD160 mRNA expression was shown to be highly restricted to NK cells and not detected in myeloid and B-cell lines by northern blot analysis.\textsuperscript{5} Outside of the immune system, CD160 is expressed on endothelial cells of neoangiogenic microvessels at the periphery of tumors.\textsuperscript{7}

NK cells play a key role in innate immunity with potent cytolytic activity against virally infected and tumor cells.\textsuperscript{8} NK cell activity is regulated by inhibitory and activatory receptors expressed at the cell surface and their interaction with associated ligands.\textsuperscript{9} CD160 binds to MHC class Ia and Ib with low affinity\textsuperscript{10} and triggers cytotoxic function in peripheral blood NK cells, as well as cytokine production, including interferon (IFN)-\gamma, tumor necrosis factor (TNF)-\alpha, and interleukin (IL)-6.\textsuperscript{11,12} Only a limited selection of human activating NK cell receptors have been demonstrated to induce cytokine production and release in addition to cytotoxicity.\textsuperscript{13} The phosphatidylinositol 3-kinase (PI3K) signaling molecule is required for CD160-mediated cytokine release, with involvement of the signaling molecules Syk and ERK upstream and downstream of PI3K, respectively.\textsuperscript{14}

Recent work has shown CD160 expression in malignant human B-cells.\textsuperscript{15} CD160 expressed on the surface of B-cell chronic lymphocytic leukemia (CLL) mimicked CD160 functions in normal NK and T-cells: cellular activation; upregulation of BCL-2, BCL-XL, improved \textit{in vitro} cell survival; and cytokine production, specifically, IL-6 and IL-8. PI3K/Akt signaling was required for CD160-mediated functions in CLL cells.\textsuperscript{15} Similar “aberrant” expression of a signaling molecule, CD3-receptor-associated protein tyrosine kinase, zeta-associated protein-70 (ZAP-70), was reported in CLL.\textsuperscript{16,17} Like CD160, ZAP-70 was initially described exclusively in T-cells and NK cells,\textsuperscript{18} but subsequently detected in mature and immature human B-lymphoid malignancies,\textsuperscript{19,21} as well as normal murine and human B-cells.\textsuperscript{22,23}
In this paper, we have investigated normal and malignant human B-cells for expression of CD160. This extensive study has established that the NK cell receptor antigen CD160 shows restricted expression in the B-cell lineage to malignant versus normal B-cells. Moreover, the varying expression of CD160 can be exploited diagnostically, as shown in test and validation sets consisting of over 970 cases of B-cell lymphoproliferative disorders (B-LPD).

Material and methods

Patients and samples

This study involved a test cohort of 811 consecutive patient samples referred for investigation of B-LPD between 2002 and 2008, in which a complete analysis was performed. Standard diagnostic criteria were used to establish the diagnosis of CLL (n=600), mantle cell lymphoma (MCL, n=34), hairy cell leukemia (HCL, n = 32) and other B-LPDs (n=145, incorporating acute lymphoblastic leukemia (ALL), and other mature B-cell malignancies). Correlation with bone marrow aspirate & trephine, lymph node and spleen by flow cytometry, histology and karyotyping was performed when required. The validation cohort included 163 consecutive cases of mature B-LPD (CLL n=113, MCL n=8, HCL n=3, other B-LPD n=39). The median fluorescence intensities (MFI) for malignant populations given in this paper are generated from the validation cohort.

Non-diagnostic analyses were approved by the National Research Ethics Service, East London and the City HA Local Research Ethics Committee and written informed consent was obtained in accordance with the Declaration of Helsinki. Following informed consent, control blood samples were taken from normal healthy donors (n=30), donor bone marrow aspirates (n=6) and cord blood sampling (n=5). Where possible tissue perfusion samples were obtained including spleen (n=5), lymph-node biopsy (n=5) and tonsil biopsies (n=4) from both healthy donors and patients.

Immunophenotypic Analysis using CD160 Flow Cytometric Assay (CD160FCA)

The five-color CD160 Flow Cytometric Assay (CD160FCA) incorporated CD2-FITC (Clone S5.2), CD5-APC (Clone L17F12), CD19-PerCP (Clone 4G7), CD23-APC (Clone EBVCS-5) (BD Biosciences, Oxford UK); CD160-PE (Clone BY55; IgM isotype; Immunotech, Beckman Coulter, Marseilles, France). A combination of internal negative controls and an isotype IgM were used (Immunotech, Beckman Coulter). Whole blood samples were analyzed within 24 hours. Leukocytes (1
x10^6) were labeled with the appropriate pre-titred antibody for 15 minutes at room temperature in darkness. Erythrocyte lysis was performed using Pharm Lyse™ (Becton Dickinson, Oxford UK), a buffered ammonium chloride-based lysing solution according to the manufacturer’s recommendations, then washed in BD Cell Wash™ (BD Biosciences, Oxford UK). A minimum of 10,000 lymphocyte-gated events was acquired for each patient on a FACS Canto (BD Biosciences, UK), with data being acquired and analyzed by means of BD FACS Diva clinical software for enhanced acquisition analysis. Positivity was defined as >20% of leukemic cells expressing a given surface antigen. A sequential gating strategy for the specific identification of CD160 on CD5^+CD19^+ B-cells using multi-color flow cytometry was established (Figure 1). The BD FACSDiva software calculated the MFI of the CD2^{neg}CD5^+CD19^+CD23^+CD160^+ population.

**CD160FCA derived scores:** a mini CLL score (zero to 3) was calculated using the three markers CD5, CD23 and CD160, with one point for each marker. CD23r, a ratio of CD23 to CD5 expression, was calculated from the percentage of CD19^+ B cells expressing CD23 versus the CD19^+CD5^+ percentage. Combining these parameters - {mini CLL score x CD23r} - allowed the immunophenotypic data to be expressed as a single numerical value, the “diagnostic discriminant”.

**RNA extraction, reverse transcription, cDNA amplification (RT-PCR) and sequencing for CD160**

PBMC from EDTA anticoagulated venous blood obtained from healthy donors and patients were isolated by density gradient centrifugation (Lymphoprep, Amersham Bioscience, UK). Fresh CD19^+ B cells were isolated using a magnetic-activated cell sorter (MACS) and a CD19^+ cell isolation kit according to the manufacturers’ recommendations (MiltenyiBiotec, Bergish-Gladbach, Germany). CD19^+cell purity was shown to be >97%.

Total RNA was isolated using the Trizol reagent according to the manufacturer’s instructions (Invitrogen, Paisley, UK). For each reverse transcription, 5µg of RNA were used. Reverse transcription was performed using 500ng of an oligo-dT primer (Invitrogen, Paisley, UK) and the superscript first strand synthesis kit (Invitrogen) in a total volume of 20µl. Specific primers for the amplification of CD160 (GenBank accession: NM_007053) cDNA were designed on the basis of published sequences. The CD160 primers were as follows: BY01 (5’-TGCAGGATGCTGTTGGAACCC-3’) (forward) and BY3UN (5’-CCTGTGCCCTGTTGCATTCTTC-3’) (reverse). β-actin cDNA amplification was
performed in parallel as an internal control. The synthesis of specific cDNA fragments was achieved by using 1µl of the reverse-transcribed product according to a standard procedure (Invitrogen), in a total volume of 20µl. Each sample was subjected to denaturation (94°C, 30 sec), annealing (60°C, 30 sec), and extension (72°C, 90 sec) steps for 35 cycles. The amplified products were separated on a 1% agarose gel. For the CD160 cDNA sequencing, the open reading frame was amplified by PCR with BY01 and BY3UN primers and Taq High Fidelity (Invitrogen). PCR product was purified (qiaex II, Qiagen) and analyzed with the following primer sequences:

Forward primers BY01 (5’-TGCAGGATGCTGTTGGAACCC-3’) and BY03 (3’-TCAGCCTGAACTGAACGTAGTGCTTTCC-5’). Reverse primers BY02 (5’-CAGCTGAGACTTTAAAGGATC-3’), BY04 (3’-CACCAACACCACCTATCCCAG-5’) and BY3UN (5’-CCTGTGCCCTGTTGCATTCTTC-3’).

**Tissue Microarray and Immunohistochemistry**

Biopsy samples were obtained from patients who had given informed consent, including 88 cases of CLL, 97 of MCL prepared on Tissue Microarrays (TMA), and 2 sections of HCL, and a control group of tissue from paraffin embedded tissue samples. TMAs were prepared from paraffin embedded histological blocks. The TMAs and the sections were dewaxed twice in xylene for 5 minutes then washed in 100% ethanol followed by two initial peroxidase blocking steps for 5 minutes each. Then, tissues were heated in a pressure cooker for approximately 10 minutes containing a TRIS-citrate based buffer (Vector Laboratories Burlingame, CA), washed in running tap water and transferred back into the buffer. The slides underwent a blocking step using horse serum for 20 minutes, then air-dried. The primary CD160 antibody (clone CL1-R2, [IgG1] produced in-house) was applied at a concentration of 10µg/ml and incubated overnight at +4 degrees. Negative control samples were prepared by omitting the primary CD160 antibody. Immunohistochemical (IHC) staining was performed using a Super Sensitive™ Polymer-HRP Detection System (Biogenex, CA, USA): the slides were subjected to a Super Enhancer Reagent™ (Biogenex, CA, USA) for 20 minutes, followed by a Poly-Horseradish Peroxidase reagent conjugated to an anti-mouse for 30 minutes at room temperature. The antibody enzyme complex was then incubated with 3,3’-diaminobenzidine (DAB), then immersed and counterstained with Gills II Haematoxylin, dipped in acid/alcohol, washed in running water and
dehydrated using ethanol followed by xylene and finally mounted with a xylene based DPX mount (Sigma, UK).

**Statistical analysis**

Standard approaches were used to calculate diagnostic indices of sensitivity and specificity. Statistical analysis was performed using Stats Direct v2.7.6 (StatsDirect Ltd, Cheshire, UK) and GraphPad PRISM 5 for Macintosh (GraphPad software, CA, USA). The diagnostic odds ratio is used as a single measure of efficacy of a diagnostic test which is unaffected by prevalence and effectively compares the odds of positivity in disease (sensitivity) relative to the odds of positivity in patients without the disease (1-specificity). The ratio can range from 0 to infinity, larger numbers indicating a better test performance. Indices are quoted as the statistic (95% confidence interval). When reporting the MFI, indices are quoted as the mean (95% confidence interval of the mean).

**Results**

*Expression of CD160 on normal B-cells*

In normal peripheral blood, CD160 protein is expressed on the cell membrane of 15 - 20 % of CD2⁺ lymphocytes and the CD160 transcript is highly restricted to NK and T-cells¹-²,⁵ (and Figure 2F). To investigate the expression on normal B-cells, the B-cell hierarchy was studied from immature hematopoietic stem cells to the mature terminally differentiated plasma cell. Hematopoietic stem cells from harvest donors (n=5) were identified using a combination of CD34⁺/CD117⁺/CD38neg/dim/CD133++. The mean percentage expression of CD160 on this population was 1.7% (0.77-2.54) (Figure 2A, 2J). Similarly, immature bone marrow B-cells - reactive benign B-cell precursors such as hematogones (n=6) (CD19⁺/CD10⁺/CD34⁺/CD38⁺) and pre-B-cells lacking surface immunoglobulin (n=6) (CD19⁺/CD10⁺/CD34⁺/Ig⁻) were negative for CD160 expression 0.78% (-0.05-1.61) and 0.43% (0.09-0.76), respectively (Figures 2B, 2C and 2J).

Mature polyclonal B-cells in secondary lymphoid organs (lymph node n=5, spleen n=5, tonsil n=4) were isolated by repeated perfusion and germinal centre (GC) B-cells were identified as CD19⁺/CD38⁺/IgD⁻ expression. The mean expression of CD160 on lymph node GC cells was 0.79% (0.29-1.28) (Figures 2D, 2J); normal polyclonal B-cells isolated from spleen and tonsil biopsies also lacked CD160 with a mean expression of 0.39% (0.20-0.60%) (Figure 2J).
Umbilical cord blood (UCB) is rich in naïve B-cells (CD19^CD5^ B1-cells). UCB B1-cells did not express CD160 (0.68%, (0.24-1.11%)) (Figures 2E, 2J). Progressing through the B-cell hierarchy, CD160 was expressed in 2.08% (1.21-2.95) peripheral blood total B-cells (Figure 2G) and 0.37% (0.16-0.58) B1-cells (Figure 2H). As a percentage of the total leukocyte population, CD160 expression on total B-cells was 0.11% (0.04-0.18). Finally, CD45^{neg/dim}/CD19^{neg}/CD38^+/CD138^+ terminally differentiated plasma cells (n=5) from normal bone marrow failed to express CD160 (0.73%, (-2.14-3.61)) (Figure 2I). There were no significant differences within the B-cell hierarchy with regard to percentage CD160 expression (P>0.05) (Figure 2J).

Circulating NK and T-cells acted as a positive control representing 11.05% of all CD2+ events (CI: 7.90-14.20). Of the NK-cells, 60.21% expressed the CD160 antigen with a MFI of 806.2 (CI: 701.8-910.5) (Figure 3A). The MFI of CD160 on immature B-cells – hematopoietic stem cells (75.3, CI: 35.36-115.3), hematogones (17.2, CI: -4.74-39.07) and pre-B-cells (26.5, CI: -0.46 – 53.46) - isolated from normal donors was significantly less than normal NK and T cells (P<0.0001) (Figure 3A).

Similarly, mature B-cell populations in both tissue and peripheral blood had low CD160 MFIs: GC B-cells 38.8 (CI: 23.06-54.54); naïve UCB B-cells 121.3 (CI: 29.2-213.3); circulating B-cells - B1-cells 76.53 (53.7-99.4) and CD5-negative B-cells 69.26 (52.2-86.4). Normal bone marrow plasma cells also had a low CD160 MFI (112.3, (84.9-139.8)) (n=5) (Figure 3A).

**Expression of CD160 in malignant B-cells**

Malignant B-cells at different stages of maturation were analyzed for CD160 protein and mRNA. Immature progenitor cells of pre-B-acute lymphoblastic leukemia (Pre-B ALL) were negative for CD160 expression (n=5) (Figure 3D). A test cohort of 811 cases of mature B-LPD with same day analysis of CD160 expression was investigated during the study period. Within the test cohort, 600 cases were diagnosed as CLL using standard criteria, of which 590 (98.3%) expressed CD160, with mean percentage CD160 positivity of 65.9%. All 32/32 cases of HCL were positive for the CD160 antigen (Figure 3H) with a mean percentage positivity of 67.8%, but a higher MFI than CLL (857.2 vs. 552.5, P=0.01). Malignant B-cells of CLL and HCL had a characteristic weak to weak-moderate staining for CD160 with a single displaced peak showing a Gaussian distribution of staining intensity. Of the 179 other B-LPD cases, 28 (15.6%) were positive for the CD160 antigen. These B-LPDs included 34 cases of MCL in leukemic phase with 29 (85.3%) negative and 5 (14.7%) positive for
CD160. Representative cases of CD160-negative immature Pre-B ALL (Figure 3D), follicular lymphoma (Figure 3E), and MCL (Figure 3F) are shown with CD160 representing <1% of the malignant population.

To confirm the flow cytometric protein expression data, total RNA was extracted from highly purified CD19+ B-cells. As expected, CD160 transcript was detected in all control mononuclear cell (MNC) fractions (representing normal NK and T-cells), but was absent from purified normal B-cells (Figure 3C), Pre-B-ALL (Figure 3D), follicular lymphoma (Figure 3E) and MCL (Figure 3F) samples. To control for contaminating normal NK and T-cells in the purified CD19+ fraction, a CD2 PCR control was used, which was negative in all cases (data not shown). CD160 transcript was only detected in purified B-cells of CLL (Figure 3G) and HCL (Figure 3H). Sequence analysis of the CD160 transcript in CLL cells corresponded to the CD160 coding sequence previously published (GenBank N°: NM_007053). These findings indicate that CD160 protein and RNA expression are absent in normal human B cells and the majority of mature B-cell malignancies, but expressed in CLL and HCL cells.

With respect to the intensity of CD160 expression, the non-CLL and non-HCL cases were grouped together for statistical comparison as ‘Non-CLL’ (Figure 3B), including cases of myeloma and Waldenström's macroglobulinemia. Circulating CLL and HCL cells had significantly higher CD160 MFIs (552.2 and 857.2 respectively) than all other sample types (76.82) (P<0.0001), including CLL tissue samples (318.8 (215.4-422.2)). The processing of CLL tissue biopsies resulted in a significant decrease in MFI expression versus PB CD160 expression, as shown by paired PB and tissue mononuclear cell suspension samples (P=0.04) (Figure 3B). Despite this decrease in expression, the MFI of the CLL tissue preparations was significantly higher than the non-CLL PB, non-CLL tissue samples and normal polyclonal B1-cells from patients with CD5-negative B-LPDs (P<0.0001) (Figure 3B). Non-CLL/non-HCL samples had CD160 MFIs equivalent to normal donor B-cells (P=0.78).

Throughout the study period CD160 analysis was performed on a number of cases of reactive polyclonal B cell lymphocytoses - in all cases the polyclonal B-cells were negative for CD160 expression.
Immunohistochemistry with CD160 in CLL, HCL and MCL

To further establish the expression of CD160 in malignant B-cells, immunohistochemistry staining of CD160 was performed using paraffin embedded histological sections and tissue microarrays (Figure 4). Of all 88 TMA CLL cases, 85 (96.5%) were positive for CD160 (Figure 4D-F). Similarly, 2 out of 2 cases of HCL (a trephine biopsy, Figure 4J; a splenic biopsy, figure 4K-L) were positive for CD160 and showed a stronger expression than in CLL, confirming the results observed by flow cytometry. Of 97 MCL cases on TMAs, only 3 cases (3%) were positive for CD160 (Figure 4 G-I).

Implementing the CD160FCA into clinical diagnostics

From a test cohort of 811 consecutive cases of mature B-LPD, a “mini CLL score” was developed using the three most consistently expressed markers in CLL - CD5, CD23 and CD160 - with each marker scoring one point. A score of 3 identified 586/600 of all cases of CLL (sensitivity = 0.98). Only 6/211 non-CLL cases scored 3 (false positive rate = 0.03) (Figure 5A): one case of HCL and 5 cases of CD23+CD160+ MCL in leukemic phase. A mini score of 3 had a very high diagnostic odds ratio of 1430 (CI: 542-3772) for CLL, with a positive predictive value of 0.99. While HCL rarely causes diagnostic confusion with CLL, morphological and immunophenotypical overlap occurs between CLL and MCL in the blood. The latter is characterized by: lymphocytes with clefted nuclei (often giving a fish-mouth appearance, colloquially referred to as ‘codocytes’), nucleoli and pleomorphism; immunophenotypically, CD5+CD23negsIgM+ (strong); and confirmed by the presence of t(11;14). Although MCL is typically CD23 negative, in the test cohort 11/34 (32 %) cases of MCL expressed CD23 (with all cases were confirmed to be MCL by G banding, fluorescence in-situ hybridization and/or histology), which is similar to other reports.26,27 In contrast, 598/600 (>99 %) cases of CLL were CD23+. As the CD23 percentage positivity in MCL is lower than in CLL, we investigated the ratio of the percentage CD23 to CD5 expression on the malignant B cells (referred to as, CD23r) as a parameter to differentiate CLL from CD23+ MCL. In CD23+ MCL cases, 87% had a ratio of 0.80 or less, with 85% of these having a ratio of 0.50 or less. In contrast, of the CLL cases analyzed (n = 431), CD23r was greater than 0.80 in 97%, and only 0.8% of CLL patients had a ratio of 0.50 or less. The disease-specific expression of the CD5/CD23/CD160 combination was also investigated using the CD160FCA methodology in a validation cohort (n=163). This confirmed CD160 positivity in CLL (107/113 cases, 95%) and the utility of the mini CLL score: 3 was diagnostic of CLL and excluded all
non-CLL cases ($P<0.0001$; sensitivity 0.95, CI: 0.89-0.98; specificity 1.00, CI: 0.93-1.00; diagnostic odds ratio of 1670, CI: 92.23-30253); while a score of 0 excluded CLL, MCL and HCL (Figure 5B). Further differentiation between B-LPDs was achieved by multiplying the mini score by CD23r to give a single numerical value, the diagnostic discriminant (Figure 5C). The diagnostic discriminant allowed immunophenotypic separation of CLL from MCL (including leukemic CD23+ MCL), HCL and other CD5+ B-LPD.

**Monoclonal B-cell lymphocytosis**

Monoclonal B-cell lymphocytosis (MBL) has been used to describe asymptomatic patients with a monoclonal lymphocytosis but $<$5000 B-cells/$\mu$l with a CLL phenotype.\textsuperscript{24} Within the validation cohort, 13.3\% (16/113) of CLL cases had a lymphocyte count $<$5x10\textsuperscript{9}/L, of which 15/16 cases expressed the CD160 antigen. The mean percentage of CD160 expression in these 16 cases was 59.5\%, similar to that of all CLL in the validation cohort (54.6\%) (Supplemental Figure 1A). Similarly, the MFI of CD160 in the 16 cases was almost identical to that of all CLL within the validation cohort (537.6 and 552.2, respectively; $P$=NS) (Supplemental Figure 1B).

**Discussion**

CD160 is a cysteine-rich, glycosylphosphatidylinositol-linked membrane protein with a single Ig-like domain and has weak homology to the firstIg-C2 domain of NK receptor, KIR2DL4: 22\% identity and 44\% similarity.\textsuperscript{5} CD160 has a broad specificity for both classical and non-classical MHC class I molecules\textsuperscript{10} and is a ligand for herpes virus entry mediator (HVEM).\textsuperscript{28} CD160 is expressed on the majority of circulating NK cells, a subset of cytotoxic T-cells, most TCR\textgreek{\gamma}\textgreek{\delta} lymphocytes, a minor subset of circulating CD8\textsuperscript{bright} TCR\textalpha\textbeta cells and all intestinal intraepithelial T lymphocytes.\textsuperscript{1-2,5} In this report, we confirm the presence of CD160 protein on circulating NK and T-cells by both flow cytometry and qualitative PCR, with CD160 expressing NK and T-cells representing 11\% of the total lymphoid population with an MFI significantly higher than other normal lymphocytes ($P<0.0001$) (Figure 3A). No CD160 expression is detectable in normal B-cells, regardless of developmental stage and the tissue of origin. From hematopoietic stem cells through to terminally differentiated plasma cells in normal donor bone marrow – via immature B-cells (hematogones and pre-B cells in bone marrow), circulating mature B-cells and naïve CD5\textsuperscript{+} B1-cells, umbilical cord blood B-cells (rich in
CD5+ B1-cells) and lymph node germinal center B cells - CD160 protein and mRNA were not detected (Figure 2). Thus, in normal immune cells, CD160 positivity is seen in NK and T-cells, but not B-cells. To date, there is no report on the expression of CD160 in malignant B-cells of chronic B-LPDs. From the test and validation sets totalling 974 patients, we were able to show almost universal expression of CD160 in CLL and HCL, while there was sporadic CD160 positivity in other B-LPDs (Figure 3). The restricted expression of CD160 in malignant, as opposed to normal, B-cells indicates it is a tumor-specific marker for B-cell malignancies. In normal immune cells, CD160 triggering leads to a variety of functional effects: cytotoxicity and cytokine secretion (IFN-γ, TNF-α, IL-6) by circulating NK cells, which is regulated by HLA-C; enhancement of CD3-induced proliferation of T-cells as a coreceptor in TCR signal transduction. Furthermore, in cytotoxic CD8+ T-cells, functional cytotoxic activity is limited to those cells that express CD160. These observations raise the possibility that the aberrant expression of CD160 in malignant B-cells may play a role in the pathophysiological process, rather than simply being a marker of malignant transformation. In CLL cells, CD160 was found to mimic the functions described in normal NK and CD8+ T-cells: cellular activation; improved in vitro cell survival; and cytokine production (IL-6 and IL-8). Moreover, PI3K/Akt signaling was required for CD160-mediated functions in CLL cells, similar to that described in normal immune cells, where Syk and ERK are involved upstream and downstream of PI3K, respectively. Thus, malignant B-cells can potentially use aberrant CD160 expression to enhance survival and cellular activation using the same intracellular pathways as normal NK and T-cells.

The “aberrant” expression of many proteins in CLL has been described – such as, the pan T-cell marker CD5 and the TCR-signal transduction molecule, ZAP-70, initially described exclusively in T-cells and NK cells. The recruitment and phosphorylation of Syk/ZAP-70 tyrosine kinases to the TCR allows differentiation and proliferation. Similar responses were shown in ZAP-70 expressing CLL cells, with enhanced signal transduction by the BCR. In the B-cell lineage, ZAP-70 was initially thought to be a tumor-specific antigen, but subsequent work found that ZAP-70 is not specific to malignant B-cells, but is also expressed in activated normal human B-cells, naive, germinal centre, memory, cord blood cells and peripheral blood B-cells. Unlike ZAP-70, CD160 expression in malignant B-cells appears to be truly “aberrant” and we have exploited this to look at its expression profile and diagnostic utility in acute and chronic B-cell malignancies. In a test cohort of 811 cases, CD160 expression was almost universal in CLL and HCL. This was confirmed in a validation cohort of
163 B-LPDs. Lymph node and splenic tissue from patients with CLL and HCL were also CD160 positive. Diagnostic immunophenotyping of B-LPDs requires a panel of MoAbs, with an ever increasing range of target antigens proposed to help differentiate between the different diseases. However, the disease-specific CD5/CD23/CD160 combination allowed a simplified “mini CLL score” to be derived from the single tube CD160FCA assay. A mini score of 3 differentiated CLL from other B-LPD, with a very high diagnostic odds ratio of 1430 (542-3772), a PPV and NPV of 99% and 94%, respectively; while a score of 0 excluded CLL, MCL and HCL. However, biological heterogeneity includes rare CD23-negative cases of CLL, while CD23 positivity in MCL is not uncommon.

In our test and validation cohorts, 13/42 (31%) cases of MCL in leukemic phase were CD23+, while 5 MCL cases in the test cohort had a mini CLL score of 3. The diagnostic discriminant was useful in such cases with an atypical immunophenotype by combining the information from the CD160FCA on the mini CLL score and the level of CD23 expression (Figure 5C). In the validation cohort, the diagnostic discriminant– (mini score x CD23r) – was ≥0.50 in all CLL cases and differentiated CLL from leukemic CD23+ MCL and other CD5+ B-LPD (P<0.0001) (Figure 5C). We propose the addition of anti-CD160 MoAb to existing diagnostic panels, with the diagnostic discriminant giving an additional robust, numerical summation of immunophenotypic data to aid in the diagnosis of B-LPD, particularly cases that are immunologically atypical. Our data suggests a simpler scoring system than both historical and current systems in place. Other molecules have recently been reported to show CLL-restricted expression, including the tumor associated antigen Receptor tyrosine-kinase Orphan Receptor 1 (ROR1) and CD200. ROR1 mRNA and surface protein were found to be strongly expressed in MCL, as well as positivity in marginal zone lymphoma, B-ALL and a subset of normal B-cell precursors. CD200 has an expression profile including CD19+ B-cells (normal and malignant), T-cell blasts, follicular dendritic cells, thymocytes, neural tissue and endothelium. Despite its expression in normal tissue, CD200 is up-regulated in CLL resulting in a down-regulation of the Th1 immune response. The weak or absent expression of CD200 in MCL has been clinically useful in the differentiation between CLL and MCL. CD160 expression shown in this paper is unique compared to both ROR1 and CD200, with absent CD160 expression in normal B-cells and specificity for CLL and HCL in malignant B-cells.

This paper demonstrates the restricted expression of the Ig-like activating NK cell receptor, CD160, in normal immune cells to NK and T-cells. Neither protein nor transcript for CD160 is found in normal B-
cells, from immature bone marrow precursors through to mature peripheral blood and germinal center B-cells. In the B-cell lineage, the restriction of CD160 expression to malignant B-cells indicates that it is a tumor specific antigen and an attractive target for minimal residual disease assessment in CD160+ B-LPD. The CD160 molecule is functional in malignant B-cells and delivers survival and activation signals to CLL cells,\textsuperscript{15} as well as CD160+ cells of SMZL and HCL-variant (data not shown), recapitulating signalling events in normal NK and T-cells. The known ligands for CD160 are expressed by both malignant B-cells themselves and other cells in the lymphoid microenvironment: MHC class I,\textsuperscript{10} CD1d,\textsuperscript{46} HLA-G\textsuperscript{10,47} and herpes virus entry mediator.\textsuperscript{6} Thus, CD160 interactions with its ligands may be important in the pathophysiology of malignant B-cells, via autocrine, paracrine and/or stromal cell interactions, offering new targets for therapeutic manipulation.

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**Authorship:**

Contribution: TWF, JG, AB, and SGA designed the research; TWF, JG and SGA performed the research, analysed the data and wrote the paper. FTL, DAT, MGM, JDC, HEO, DT, ACN, MC, MJ and JGG performed the research and co-authored; AB provided new reagents.

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References


**Figure legends**

**Figure 1: Sequential gating strategy for CD160 detection on malignant CD19+ B-cells.** Total B cells were identified using: (A) forward and side scatter to gate the lymphoid region and exclude any apoptotic cells and debris. From this (B) CD19+ versus side scatter, to exclude any non-specific binding. (C) CD19+ B-cells were further isolated by gating the CD2+ events and generating a ‘NOT’ (exclusion) gate. (D) The malignant B-cells were separated from the normal residual B cells using a CD2negCD5+CD19+ gate. (E) CD23 expression was calculated from the malignant population and used to generate the mini CLL score and CD23r. (F) In the context of CLL, CD160 positivity defined on the CD2negCD5+CD19+CD23+ population was then calculated from this ‘pure’ malignant B-cell population.

**Figure 2: CD160 expression in normal lymphocytes is restricted to NK and T-cells.**

(A) CD160 expression on normal donor hematopoietic stem cells (HSC) gated on the CD34+CD117+ cells (n=5). (B) CD160 expression on immature reactive benign B-cell precursors, termed hematogones, that are typically CD19+CD10+CD34+CD38+ (n=5). (C) CD160 expression on pre-B cells from bone marrow samples which lack surface immunoglobulin but are CD19+CD10+CD34neg (n=6). (D) CD19+CD38+IgDneg germinal centre cells from both tonsil and lymph node (n=6). (E) Umbilical cord blood (UCB), showing CD160 and CD19 expression on CD5+ cells (n=5). (F) Normal peripheral blood NK cells demonstrating CD160 positivity (n=5). (G) Normal peripheral blood mononuclear B-cells (n=5). (H) Normal B1-cells from a healthy donor (n=5). (I) CD45negdim CD38+CD138+ terminally differentiated plasma cells from bone marrow samples (n=5). (J) Percentage CD160 expressed on individual cell types: HSC – hematopoietic stem cells; BM – bone marrow; PB – peripheral blood; UCB – umbilical cord blood; LNBx – lymph node biopsy; GC – germinal centre; Secondary lymphoid organs – splenic and tonsillar material).
Figure 3: **CD160 protein and transcript expression is restricted to CLL and HCL.**


(B) MFI of malignant B-cells demonstrating significantly increased expression in CLL and HCL. ‘non-CLL’ represents all B-LPD, excluding CLL and HCL cases. All tissue fields include lymph node, spleen, bone marrow and tonsilar material. Non-CLL Tissue includes pre-B-ALL bone marrow samples. *: P<0.0001, **: P<0.0001, ***: P=0.0114 (C-H) Representative flow cytometric images of (C) normal PBMC, (D) Pre-B acute lymphoblastic leukaemia, (E) Follicular Lymphoma (F) mantle cell lymphoma, (G) CLL and (H) HCL. Below each flow cytometric plot is shown the corresponding cDNA amplification using specific primers for CD160 following reverse transcription of total RNA extracted from highly purified CD19+ B cells (isolated using a magnetic-activated cell sorter; purity > 97%). β actin cDNA synthesis was used as internal control.

Figure 4: **Immunohistological staining of CD160 in chronic B-cell malignancies**

Tissue sections and TMAs were stained with CD160 antibody (10μg/ml), incubated overnight at +4 degrees and revealed using the Super Sensitive™ Polymer-HRP Detection System (Biogenex). (A-C) Control: a lymph node TMA spot from a patient with CLL without the primary antibody (objective: A x5, B x20, C x63). (D-F) Positive CD160 staining of a CLL lymph node TMA (objective: A x5, B x20, C x63). Of CLL TMA cases, 85/88 (96.5%) were CD160 positive. (G-I) Negative CD160 staining of a MCL lymph node on a TMA (objective: A x5, B x20, C x63). Of MCL TMA cases, 94/97 cases were CD160 negative. (J-L) HCL: 2 cases showing typical strong CD160 positivity; a bone marrow trephine (J) and a splenic biopsy (objective: K x43, L x63).

Figure 5: **Mini CLL Score for B-cell lymphoproliferative disorders and the generation of the ‘Diagnostic Discriminant’ to differentiate between CLL and MCL.**

The mini CLL score was derived from the CD160FCA, with one point for each marker – CD5, CD23 and CD160. (A) The mini CLL score within the test cohort (n=811). (B) The mini CLL score within the validation cohort (n=163). (C) The diagnostic discriminant was generated by multiplying the mini CLL score and CD23r variables. CLL versus MCL (*: P<0.0001), HCL (**P=0.0002) and CD5+ chronic B-cell malignancies (***P<0.0001).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Differential and tumor-specific expression of CD160 in B-cell malignancies

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