Somatic Activating Mutations In CXCR4 Are Common In Patients With Waldenstrom’s Macroglobulinemia, and Their Expression In WM Cells Promotes Resistance To Ibrutinib

Yang Cao, Zachary Hunter, Xiao Liu, Lian Xue, Guang Yang, Christina K Tripsas, Robert Manning, Christopher J. Patterson, Steven P. Teeron, Bing Center for Waldenstrom’s Macroglobulinemia, Dana Farber Cancer Institute, and Harvard Medical School, Boston, MA, USA.

Background: Waldenstrom’s macroglobulinemia (WM) is an indolent non-Hodgkin’s lymphoma characterized by the accumulation of IgM-secreting lymphoplasmacytic cells in the bone marrow. CXCR4 is a chemokine receptor that promotes the survival, migration, and adhesion to the bone marrow stroma of WM lymphoplasmacytic cells. CXCR4 is also expressed in a variety of cancers, and loss of its interactions with its ligand CXCL12. Through whole genome sequencing, we identified somatic mutations in CXCR4 that affected 1/3 of WM patients. These mutations were identical to those identified in Waldenstrom Macroglobulinemia (WHIM) syndrome, and in Myelodysplastic Syndrome (MDS). In WHIM syndrome, loss of the c-terminal tail of CXCR4 impairs receptor internalization, thereby prolonging G-protein and B-arrestin signaling (Langan et al., Blood, 2008). Ibrutinib induces WM cell death, and is highly active in WM (Teeron et al, ICLM-12, 2013). Since the target of Ibrutinib (BTK) is a known downstream target of CXCR4 and triggers ERK and Akt activity, we sought to clarify if ibritunib activity in WM LPCs was modulated by WHIM-like mutations in CXCR4.

Methods: We first sought to confirm the frequency of WHIM-like mutations in 87 untreated WM patients by Sanger sequencing. The most common CXCR4 somatic mutation identified in these studies, S338X, was then cloned by PCR from CD19+ LPCs from a WM patient with this identical somatic mutation. Wild type (WT) and S338X CXCR4 cDNA were subcloned into pIRES-GFP vector, and transduced using an optimized lentiviral strategy into BCWM1 WM cells. Five days after transduction, GFP positive cells were sorted and used for functional studies. Surface expression of CXCR4 was determined by flow cytometric analysis using a PE-conjugated anti-CXCR4 monoclonal antibody. The expression of phosphorylated BTK, AKT, and ERK1/2 was determined by phospho-flow analysis and confirmed by western blotting. Cell proliferation was measured by Alamar Blue assay.

Results: Sanger sequencing identified identical somatic nonsense or frame shift mutations (WHIM-like) in the c-terminal tail of CXCR4 in 28 of 87 (32%) WM patients many of which were identical to germline variants found in WHIM syndrome (Figure 1). BCWM1 cells were then transduced with wild type CXCR4 or S338X mutant expression vectors. Expression was confirmed by cDNA Sanger sequencing. CXCR4 S338X expressing cells showed enhanced and prolonged phosphorylated ERK, AKT and BTK activity versus wild type controls. Cells transduced in the presence of SD1a (Figure 2). Ibrutinib treated control vector and CXCR4 wild-type transduced cells showed suppressed tumor cell growth in the presence of the CXCR4 ligand CXCL12 (20 nM), whereas cells transduced with CXCR4 WT expressed like CXCR4 WT. Ibrutinib induced mutation demonstrated resistance to ibrutinib mediated growth suppression (Figure 3; p<0.01). In turn, this rescue could be blocked by treatment with 30uM of the specific CXCR4 inhibitor AD3100 confirming that this effect was mediated through CXCR4 (p<0.01) (Figure 2). Phosphorylated ERK1/2 and AKT signaling increased following CXCL12 stimulation in all cells lines, though enhanced and extended activity was observed in BCWM1 WT cells and S338X mutant cells. CXCR4 triggered signaling by CXCL12 in these experiments was confirmed by pre-treatment with AD3100 (Figure 4).

Conclusions: By Sanger sequencing, WHIM-like CXCR4 somatic mutations are observed in 32% of untreated WM patients, and promote ERK, AKT, and BTK activity in the presence of SD1a. WHIM-like CXCR4 mutations are associated with resistance to ibrutinib, and mediate ERK1/2 and AKT signaling in the presence of the CXCR4 ligand CXCL12 in WM cells. These studies have important implications for CXCR4 modulation in the treatment of WM, as well as potential use of CXCR4 mutations in predicting outcome for patients undergoing ibrutinib therapy.

Figure 1: Identification of novel CXCR4 mutations in WM disease. We performed whole genome sequencing using paired genome WM lymphoplasmacytic (LPL) cells and identified somatic CXCR4 mutations in 8 out of 30 (27%) WM patients. The results were validated by Sanger sequencing. We further screened 87 untreated patients, 28 (32%) of whom exhibited CXCR4 C-terminal mutations. (A) CXCR4 is a G-protein-coupled receptor, which is expressed in the N-terminal extracellular region, three extracellular loop (ECL), seven transmembrane regions and a C-terminal tail. All identified mutations are located in C-terminal mutations in CXCR4 were reported in the germline of patients with WHIM (Warts, Hypogammaglobulinemia, Infertility and Myelodysplasia Syndrome), a dominant autosomal genetic disorder caused by tonic CXCR4 activation. These C-terminal truncating mutations have been shown to impair CXCR4 internalization resulting in sustained G-protein-dependent responses, and chemotaxis. (B) Summary of CXCR4 mutations found in WM patients. The most common mutation is S338X. Among all WM patients with a WHIM-CXCR4 mutation, S338X occurs in 42%.

Figure 2: Phospho-flow analysis for phosphoryl-ERK, AKT, and BTK in CXCR4 transduced BCWM1. 1 WM cell lines. Phospho-vector (Vec), wild type CXCR4 (WT) and mutant C1013G (Mut) C1013G transduced BCWM1-F cell line were pre-treated with 4 hrs before stimulation with SD1a (10uM). Cells were fixed with BD Phosflow Fix Buffer at the indicated time point at 37° for 10mins followed by wash twice with 1% para/wash buffer i. FACs analysis using conjugated anti-phospho-ERK1/2 (Y202/204) and anti-phospho-Akt(Y473) antibodies, and phospho-BTK (pY223) antibody (BD Phosflow). Data represent the mean ± SEM. **p<0.05, ***p<0.01, ****p<0.001, mutant vs. wt: n= 3 for pERK and pAKT, n= 6 for pBTK. Results were confirmed by western blot analysis.

Figure 3: Assessment of CXCL12 (SFDA) induced growth on vector only, wild type CXCR4, and WHIM-like mutant CXCR4 in BCWM1 WM cells following Ibrutinib (PCI-32275) treatment. These studies depict that S338X transduced BCWM1 patient cells were resistant to the inhibitory effects of Ibrutinib on cell growth in the presence of SD1a. Transfected cells were treated with Ibrutinib (0.5uM or 1uM) alone, or Ibrutinib plus SD1a (10uM), or Ibrutinib plus SD1a (20nM) and AM100 (50uM) for 24hrs. Cell proliferation was measured with Alamar blue (Invitrogen). p-value < 0.01 for CXCL12 rescue vs. PCI alone or PCI + AM100 for both 0.5uM and 1uM doses in cells transduced with the WHIM-like CXCR4 mutation. Graph represents median values and ranges with experiments conducted in quadruplicate.