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

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Abstract

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 (LPC) through 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 or functionally similar to those associated with *Warts, Hypogammaglobulinemia, Infection, and Myelokathexis* (WHIM) syndrome (Hunter et al, ASCO 2012), a rare autosomal dominant genetic disorder that is caused by frame shift or nonsense mutations in the carboxyl-terminal cytoplasmic tail of CXCR4. In WHIM syndrome, loss of the c-terminal tail of CXCR4 impairs receptor internalization, thereby prolonging G-protein and β-arrestin signaling (Lagane et al, Blood 2008). Ibrutinib induces WM cell death, and is highly active in WM (Treon et al, ICML-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 ibrutinib 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 somatic mutation. Wild type (WT) and S338X CXCR4 cDNAs were subcloned into plenti-IRES-GFP vector, and transduced using an optimized lentiviral based strategy into BCWM.1 WM cells. Five days after transduction, GFP positive cells were sorted and used for functional studies. Surface expression of CXCR4 was determined by flow cytometeric 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 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). BCWM.1 cells were then transduced with control vector, CXCR4 wild type or CXCR4 S338X mutant expressing vectors. Expression was confirmed by cDNA Sanger sequencing. CXCR4 S338X expressing cells showed enhanced and prolonged phospho-ERK, AKT and BTK activity versus wild type and vector transfected cells in the presence of SDF1a (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 S338X WHIM-like 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 CXCR4 specific inhibitor AMD3100 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 exhibited enhanced and extended activity in S338X transduced cells (Figure 3). Ibrutinib inhibited BTK, ERK1/2 and AKT activation with or without CXCL12 stimulation in control vector and CXCR4 wild-type, but not CXCR4 S338X mutant cells. CXCR4 triggered signaling by CXCL12 in these experiments was confirmed by pre-treatment with AMD3100. (Figure 4)

Conclusions: By Sanger sequencing, WHIM-like CXCR4 somatic mutations are observed in 28/87 (32%) of untreated WM patients, and promote ERK, AKT, and BTK activity in the presence of SDF1a. 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.

Disclosures: Dr. Treon received research funding, consulting fees, and honoraria from Pharmacyclics and Jannsen Pharmaceutics which market Ibrutinib.

Results

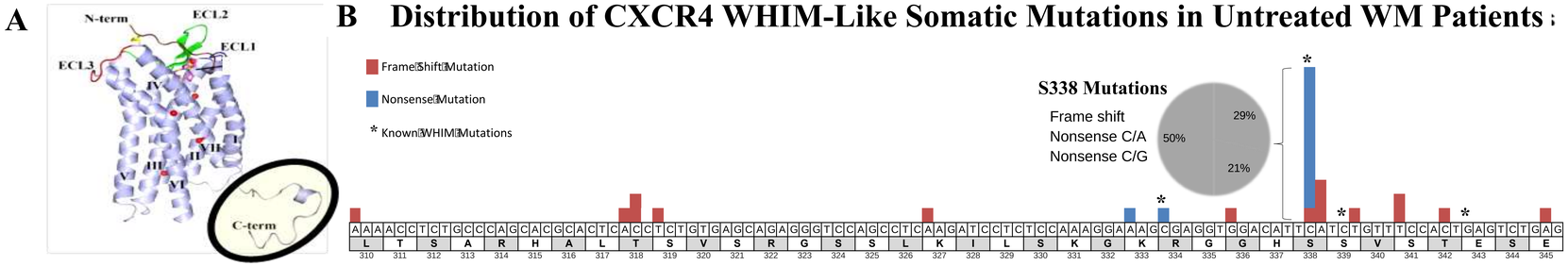

Figure 1: Identification of novel CXCR4 mutations in WM disease. We performed whole genome sequencing using paired germline/ WM lymphoplasmacytic (LPL) cells and identified somatic CXCR4 mutations in 8 out of 30 (27%) WM patients. The results were validated by Sanger sequencing. We further screened 87 untreated patients, 28 (32%) of whom exhibited CXCR4 C-terminal mutations. (**A**) CXCR4 is a G-protein-coupled receptor with an N-terminal extracelluar region, three extracellular loop (ECL), seven transmembrane regions and a C-terminal tail. All identified mutations are located in C-terminal of CXCR4. Similar C-terminal mutations in CXCR4 were reported in the germline of patients with WHIM (Warts, Hypogammaglobulinemia, Infections and Myelokathexis) 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%.

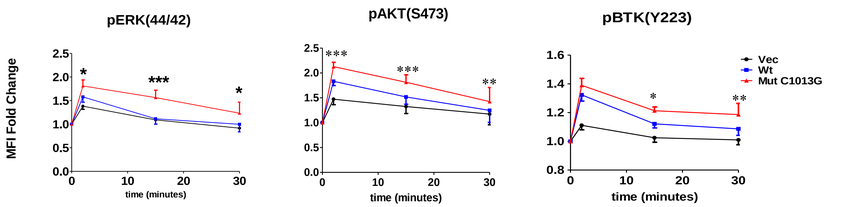

Figure 2. Phospho-flow analysis for phospho-ERK, AKT, and BTK in CXCR4 transfected BCWM. 1 WM cell lines. Plenti-vector (Vec), wild type CXCR4 (Wt) and mutant C1013G (Mut C1013G) transduced BCWM1-EP cell line were starved for 4 hrs before stimulation with SDF1α (10nM). Cells were fixed with BD Phosflow Fix Buffer1 at the indicated time point at 37°c for 10mins followed by wash twice with 1 × perm/wash buffer I. FACS analysis using conjugated anti-phospho-ERK1/2(pT202/pY204) and anti-phospho-AKT(pS473) antibodies, and phospho-BTK (pY223) anibody (BD Phosflow). Data represent the mean +/- SEM. *P<0.05, **P<0.01, ***P<.001, mutant vs wt. n= 3 for pERK and pAKT, n=6 for pBTK. Results were confirmed by western blot analysis.

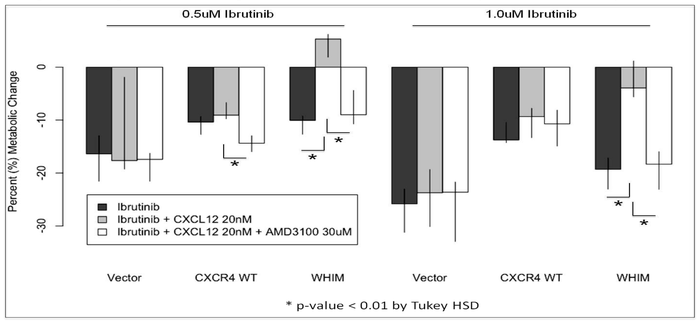

Figure 3. Assessment of CXCL12 (SDF1a) induced growth on vector only, wild-type CXCR4, and WHIM-mutation (S338X) transfected BCWM.1 WM cells following Ibrutinib (PCI-32765) treatment. These studies depict that S338X transfected BCWM.1 WM cells were resistant to the inhibitory effects of Ibrutinib on cell growth in the presence of SDF1a. Tranfected cells were treated with Ibrutinib (0.5uM or 1uM) alone, or Ibrutinib plus SDF1a (20nM), or Ibutinib plus SDF1α (20nM) and AMD3100 (30uM) for 24hrs. Cell proliferation were measured with Alamar blue (Invitrogene). * p-value < 0.01 for CXCL12 rescue vs. PCI alone or PCI + AMD3100 for both 0.5uM and 1uM doses in WM cells transduced with the WHIM-like CXCR4 mutation. Graph represents median values and ranges with experiments conducted in quadruplicate.

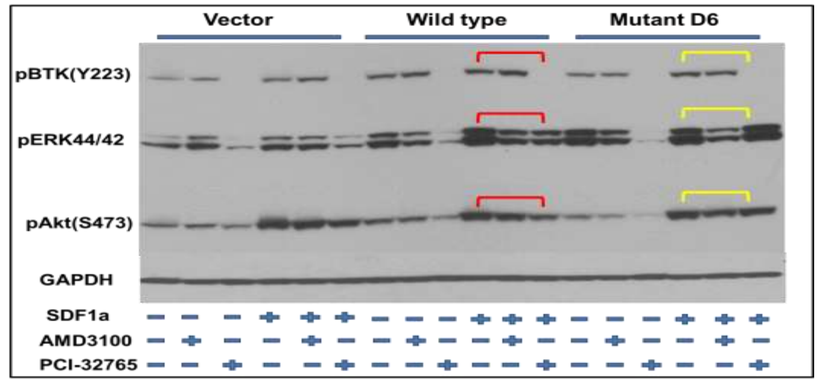

Figure 4. Mutant CXCR4 transduced BCWM.1 WM cells exhibit resistance to Ibrutinib (PCI-32765) mediated suppression of ERK1/2 and AKT activation. Plenti-vector, wild type CXCR4 and mutant S338X transduced BCWM.1 cell lines were starved for 4 hrs and incubated with DMSO or AMD3100 30uM or Ibrutinib 0.5uM for 1.5 hrs before treatment with or without SDF1a at 10 nM. 2 minutes after SDF1a stimulation, cell lysates were collected, and immunoblotted for phospho-BTK, phospho-ERK and phospho-AKT, GAPDH was used as loading control.

Summary

- **WHIM-like CXCR4 somatic mutations were identified in 28/87 (32%) of untreated WM patients. WHIM-CXCR4 mutation (C1013G; S338X) leads to enhanced and sustained ERK, AKT, and BTK activity in the presence of SDF1a in WM cells.**
- **WHIM-like CXCR4 (C1013G) transfected WM cells show decreased sensitivity to Ibrutinib mediated suppression of ERK1/2 and AKT signaling, and to the inhibitory effects of Ibrutinib on cell growth in the presence of SDF1a. Importantly, these effects could be reversed with a specific inhibitor of CXCR4.**
- **These studies support the recent finding by Treon et al, ASH 2013 showing that CXCR4 mutations impact Ibrutinib responses in relapsed/refractory WM patients, and that CXCR4 mutation status may serve as a useful biomarker for predicting Ibrutinib activity in WM patients. Importantly, these studies also show that targeting CXCR4 via inhibitors may represent an attractive strategy for augmenting Ibrutinib activity in CXCR4 mutated WM patients, and therefore provide a framework for examining Ibrutinib and CXCR4 inhibitors in WM patients.**