MYD88 mutations can be used to identify malignant pleural effusions in Waldenström macroglobulinaemia

Malignant pleural effusions are a rare extramedullary manifestation of Waldenström macroglobulinaemia (WM) (Banwait *et al*, 2015), a B-cell malignancy characterized by bone marrow (BM) infiltration with IgM-secreting lymphoplasmacytic lymphoma. Establishing a malignant aetiology for pleural effusions in WM patients is often inconclusive with standard diagnostic tests. Cytology generally cannot distinguish between lymphocyte-rich malignant and non-malignant pleural effusions (Kavuru *et al*, 1992; Mansoor *et al*, 2000). Additionally, pleural fluid specimens are typically devoid of extensive tumour involvement (Alexandrakis *et al*, 2004), adversely impacting the sensitivity of flow cytometric and gene rearrangement studies. To ensure timely initiation of appropriate antineoplastic therapy, methods to reliably detect pleural infiltration by WM are needed.

Recent studies have identified the *MYD88* L265P mutation in >90% of WM patients, though more rarely non-L265P mutations can be present (Treon *et al*, 2012, 2015). Clinical detection of *MYD88* L265P in the BM is routinely performed using highly sensitive allele-specific polymerase chain reaction (AS-PCR) to support the diagnosis of WM (Xu *et al*, 2013). The presence of *MYD88* L265P in cerebrospinal fluid has also been shown to support the diagnosis of Bing-Neel syndrome, a rare complication of central nervous system involvement by WM cells (Poulain *et al*, 2014). Consequentially, we hypothesized that the identification of *MYD88* L265P in pleural fluid could similarly identify the presence of malignant WM cells, and establish the diagnosis of a malignant pleural effusion.

We identified 9 WM patients with pleural effusions who had *MYD88* testing performed on pleural fluid samples suspected to be malignant in nature. AS-PCR for *MYD88* L265P was performed for Patients 1-8 on both unselected pleural fluid and BM samples (Xu *et al*, 2013). Sanger sequencing of selected $CD19^+$ B-cells from pleural fluid was performed for Patient 9, who had a known *MYD88* S243N mutation. *CXCR4* mutations were screened in both the pleural fluid ($n = 3$) and BM ($n = 9$) with selected $CD19^+$ B-cells using Sanger sequencing and AS-PCR for *CXCR4* S338X in patients with adequate samples (Xu *et al*, 2015). Cytology, flow cytometry, and immunoglobulin heavy chain gene (*IGH*) rearrangement studies on pleural fluid were also performed.

The clinical presentation and diagnostic work-up for the pleural effusions is displayed in Table I. The median time from WM diagnosis to pleural effusion onset was 9 years (range 1·7–20·6 years), and all patients had received a median of 2 (range 1–6) prior therapies for WM. Three patients were on active ibrutinib therapy at the time pleural effusions developed. Malignant lymphoplasmacytic cells were identified by cytological evaluation in only two patients (2/9, 22%). Flow cytometry identified a clonal population of B-cells in the same two patients (2/9, 22%), but in none of the others. *IGH* rearrangement was performed in 7 patients, and revealed a clonal pattern in 6 patients (6/7, 86%). Mutated *MYD88* was detected in the pleural fluid sample of all 9 (100%) patients. Mutated *MYD88* (8 *MYD88* L265P, 1 *MYD88* S243N) was also identified in BM samples obtained in all patients. All but one of these patients were wild-type for *CXCR4* in the same BM samples. Selected B-cells from the pleural effusion was available for three patients (all of whom were wild-type for *CXCR4* by BM examination), and revealed the same genotype.

The detection of mutated *MYD88* in pleural fluid supported the diagnosis of a malignant pleural effusion, and the decision to treat. All 9 patients experienced clinical improvement in response to antineoplastic therapy (Table II). Four patients received ibrutinib, three patients received bendamustine alone ($N = 1$) or with an anti-CD20 monoclonal antibody ($N = 3$) and one patient received bortezomib with dexamethasone. With a median follow-up of 12·2 months (range 4·8–36·1), 8 patients achieved a complete resolution (89%), and one had stabilization of pleural effusions. All patients achieved a major systemic response to therapy.

The detection of WM cells in a pleural effusion can be a diagnostic challenge, and hinder or delay chemotherapy administration in the absence of a supportive pathological diagnosis. The differential diagnosis with lymphocyte-rich effusions can include chylothorax, tuberculosis, chronic congestive heart failure and rheumatoid disease, limiting the value of cytological examination. Use of flow cytometry or *IGH* rearrangement to detect a clonal B-cell population can help improve diagnostic certainty (Kavuru *et al*, 1992; Mansoor *et al*, 2000). However, a paucity of tumour cells due to pleural adherence or excess normal B-cells in the sample can impede the identification of a clonal population by either flow cytometry or *IGH* rearrangement assay (Mansoor *et al*, 2000; Alexandrakis *et al*, 2004). The *IGH* rearrangement assay has a lower limit of detection 2 orders of magnitude less than the AS-PCR assay used to detect *MYD88* L265P (Xu *et al*, 2013), and may explain the lack of detection in one patient who had mutated *MYD88* but did not have an identifiable *IGH* rearrangement. As such, the use of AS-PCR

Table I. Clinical presentation and diagnostic test results of patients with malignant pleural effusions.

							Pleural Fluid			*MYD88* mutation		*CXCR4* mutation	
Patient	Age (years)	Gender	Symptoms	Imaging	Presentation	Serum IgM (g/l)	Cytology	Flow cytometry	*IGH* rearrangement	Bone marrow	Pleural fluid	Bone marrow	Pleural fluid
1	72	Female	SOB, cough	Bilateral moderate to large PE	Progressive event off-therapy	19·6	No malignant cells	$CD5^+$, $CD10^-$, $CD23^+$, $CD19^+$, $CD20^+$, polytypic LC	Polyclonal pattern	Present	Present	Present (S338X)	Not available
2	54	Female	SOB	Unilateral PE on right side	Progressive event while on ibrutinib	5·5	No malignant cells	$CD5^+$, $CD10^-$, $CD23^-$, $CD19^+$, $CD20^+$, polytypic LC	Clonal pattern	Present	Present	Absent	Absent
3	73	Male	SOB, fatigue	Unilateral PE on left side	Progressive event while on ibrutinib	45·8	No malignant cells	$CD5^+$, $CD10^-$, $CD23^-$, $CD19^+$, $CD20^+$, polytypic LC	Clonal pattern	Present	Present	Absent	Not available
4	68	Male	SOB, fatigue	Bilateral PE, left > right	Progressive event off-therapy	22·1	No malignant cells	$CD5^-$, $CD10^-$, $CD23^-$, $CD19^+$, $CD20^+$, polytypic LC	Clonal pattern	Present	Present	Absent	Not available
5	70	Male	SOB, weight loss	Unilateral PE on left side	Progressive event off-therapy	24·7	No malignant cells	$CD5^-$, $CD10^-$, $CD23^-$, $CD19^+$, $CD20^+$, polytypic LC	Clonal pattern	Present	Present	Absent	Absent
6	51	Male	SOB, fatigue	Unilateral PE on left side	Progressive event off-therapy	44·2	No malignant cells	$CD5^-$, $CD10^-$, $CD23^+$, $CD19^+$, $CD20^+$, polytypic LC	Clonal pattern	Present	Present	Absent	Not available
7	75	Male	SOB	Unilateral PE on left side	Progressive event off-therapy	25·5	Malignant cells identified	$CD5^-$, $CD10^-$, $CD23^-$, $CD19^+$, $CD20^+$, monotypic λ LC	Clonal pattern	Present	Present	Absent	Absent
8	69	Male	Cough on deep inspiration	Unilateral PE on left side	Progressive event off-therapy	22·2	No malignant cells	$CD5^-$, $CD10^-$, $CD23^+$, $CD19^+$, $CD20^+$, polytypic LC	Not performed	Present	Present	Absent	Not available
9	69	Male	SOB, fatigue	Bilateral PE	Progressive event while on ibrutinib	6·1	Malignant cells identified	$CD5^+$, $CD10^+$, $CD23^-$, $CD19^+$, $CD20^+$, monotypic λ LC	Not performed	Present*	Present*	Absent	Not available

SOB, shortness of breath; PE, pleural effusion; LC, light chain.

*Patient 9 had *MYD88* S243N detected; all other patients had *MYD88* L265P identified.

Table II. Treatment and clinical response of malignant pleural effusions.

Patient	Time between PE onset and WM diagnosis (years)	Number of previous therapies	Treatment for PE	IgM response	Clinical response	Follow-up time (months)
1	3·5	1	Ibrutinib 420 mg PO qD	VGPR	Resolution of SOB, improved cough. Additional thoracentesis not required once therapy initiated. No recurrence of PE.	15·4
2	3·7	2	Bendamustine 70 mg/m^2 IV + ofatumumab IV (4 cycles)	PR	Exercising without SOB. Continued to have thoracentesis every 2–3 weeks for 3 months while on therapy.	10·0
3	16·1	6	Bortezomib 1·6 mg/m^2 SQ + dexamethasone 20 mg (4 cycles)	PR	Significant improvement in energy. Additional thoracentesis not required once therapy initiated. No recurrence of PE.	21·5
4	9·0	1	Bendamustine 90 mg/m^2 IV + rituximab 375 mg/m^2 IV (6 cycles)	PR	Complete resolution of PE after 6 months of therapy. PE recurred after 1 year, and the patient was initiated on ibrutinib 420 mg PO qD.	33·8
5	5·8	2	Ibrutinib 420 mg PO qD	PR	Resolution of SOB, and pleural catheter was removed after 4 weeks on therapy. No recurrence of PE.	12·2
6	1·7	1	Ibrutinib 420 mg PO qD	PR	Resolution of SOB and improved energy level. No recurrence of PE after therapy initiation.	36·1
7	20·6	4	Ibrutinib 420 mg PO qD	PR	Pleural catheter was inserted at time of therapy initiation, and was removed after 6 months. No recurrence of PE.	10·8
8	14·0	3	Bendamustine 70 mg/m^2 IV + rituximab 375 mg/m^2 IV (treatment ongoing at this time; patient has received 3 cycles thus far)	PR	SOB has stabilized. Patient still reports some pain in left chest upon deep inspiration.	4·8
9	3·5	2	Bendamustine 90 mg/m^2 IV (6 cycles)	PR	Improved performance status with resolution of SOB. No evidence of recurring PE.	7·4

SOB, shortness of breath; PE, pleural effusion; CR, complete response; VGPR, very good partial response; PR, partial response.

for mutated *MYD88* may represent a more sensitive tumour-directed molecular tool for supporting the diagnosis of a malignant pleural effusion in WM.

The predominance of wild-type *CXCR4* patients in this cohort is consistent with the genotype of WM patients who typically develop extramedullary disease. *CXCR4* promotes homing of the WM cells in the BM, and its absence may account for the higher rate of extramedullary disease observed among wild-type *CXCR4* patients (Treon *et al*, 2014). Extramedullary involvement also appears more prominent among previously treated WM patients further along in their disease course (Banwait *et al*, 2015), which may reflect the emergence of a clone with extramedullary preference in response to therapeutic pressure. Such clonal evolution may explain the incidence of pleural effusions described herein, as onset occurred after multiple therapies and approximately 10 years from the initial WM diagnosis.

In summary, our studies show that mutated *MYD88* can be used to identify malignant pleural effusions in WM patients. WM patients with a suspected malignant pleural effusion should be considered for *MYD88* mutation testing (*MYD88* L265P and non-*MYD88* L265P, if wild-type by AS-PCR for *MYD88* L265P) as part of their workup to establish the aetiology of their pleural effusion.

Author contributions

JNG, SPT, JJC designed the study. ZRH, LX, SPT performed MYD88 mutation testing. JNG, KM gathered clinical data. JNG drafted the initial manuscript. All the authors read and approved the final manuscript.

Joshua N. Gustine[1]
Kirsten Meid[1]
Zachary R. Hunter[1]
Lian Xu[1]
Steven P. Treon[1,2]
Jorge J. Castillo[1,2]
[1]Bing Center for Waldenström Macroglobulinemia, Dana-Farber Cancer Institute, and [2]Department of Medicine, Harvard Medical School, Boston, MA, USA.
E-mail: jorgej_castillo@dfci.harvard.edu

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Pim-2 is a critical target for treatment of osteoclastogenesis enhanced in myeloma

Multiple myeloma (MM) has a unique propensity to develop and expand almost exclusively in the bone marrow, and generates bone destruction. MM bone disease is characterized by increased osteoclastic activity and suppressed osteoblastic differentiation causing devastating bone destruction with rapid loss of bone (Roodman, 2006; Raje & Roodman, 2011). In pursuing novel factors responsible for MM tumour expansion in the bone lesions, Pim-2 (also termed PIM2) has been found to be constitutively over-expressed as an anti-apoptotic mediator in MM cells and further upregulated through an interaction with osteoclasts and/or bone marrow stromal cells with defective osteoblastic differentiation (Asano *et al*, 2011; Johrer *et al*, 2012; Lu *et al*, 2013). We subsequently reported that Pim-2 is also induced in bone marrow stromal cells in MM and acts as a negative regulator for osteoblastogenesis, and that Pim inhibition is able to restore bone formation in addition to the suppression of the tumour burden in MM animal models (Hiasa *et al*, 2015). Thus, Pim-2 appears to be an important therapeutic target in MM and clinical studies with Pim inhibitors have been launched (NCT01456689, NCT02144038). However, the effects of Pim inhibition on osteoclastogenesis enhanced in MM has not been studied. Therefore, we explored the expression of Pim-2 in osteoclastic lineage cells, its role in osteoclastogenesis, and the impact of Pim inhibition on MM-induced bone resorption. Details of the Materials and method are available in Data S1.

Pim-2 was expressed in cathepsin K-positive mature osteoclasts on the surface of bone in addition to MM cells in mouse MM models with intra-tibial inoculation of mouse 5TGM1 MM cells (Fig 1A, top). Pim-2 was also clearly expressed exclusively in cathepsin K-positive osteoclasts, but not in other bone marrow cells in the tibia of normal control mice (Fig 1A, bottom). Receptor activator of NF-κB ligand (RANKL, TNFSF11), a critical mediator of osteoclastogenesis, is overproduced to extensively enhance bone resorption in MM (Pearse *et al*, 2001; Giuliani *et al*, 2002). Addition of RANKL time-dependently induced the expression of Pim-2 in parallel with c-fos (FOS), NFATc1 (NFATC1), critical transcription factors for osteoclastogenesis and cathepsin K, a functional marker of mature osteoclasts, in RAW264.7 pre-osteoclastic cells (Fig 1B, left). Inhibition of the NF-κB pathway by addition of SN50 or IMG2001, inhibitory peptides for the nuclear translocation of p65 (RELA) or p50 (NFKB1),