

# Detection of phospho-STAT5 in mast cells: a reliable phenotypic marker of systemic mast cell disease that reflects constitutive tyrosine kinase activation

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## Summary

Systemic mastocytosis (SM) is characterized by the abnormal proliferation and accumulation of mast cells (MCs). Constitutive activation of kit, a receptor tyrosine kinase (TK), has been associated with all types of SM. Signal transducers and activators of transcription (STATs), such as STAT5, mediate downstream kit signalling. We hypothesized that nuclear phospho-STAT5 (pSTAT5) in MCs might reflect TK activation and would be a marker of abnormal MCs in SM. Expression of tryptase, CD25, CD2 and pSTAT5 was evaluated by immunohistochemistry (IHC) on archival cases of SM and cutaneous mastocytosis (CM). pSTAT5 was detected in 23/23 of SM and 1/9 of CM MC nuclei. 23/23 SM had CD25 + MCs. Control tissue MCs were negative for pSTAT5. Nuclear pSTAT5 in MCs from SM reflects abnormal TK activation. We propose nuclear pSTAT5 positivity in MCs as an additional minor phenotypic criterion for diagnosis of SM in future World Health Organization classification schemes.

**Keywords:** systemic mast cell disease, mastocytosis, kit, STAT5.

Mastocytosis syndromes are currently divided into cutaneous mastocytosis (CM) and systemic mastocytosis (SM). The former generally occurs in childhood and has a self-limited course, whereas the latter more often occurs in adulthood and may or may not be associated with other non-mast cell (MC) haematological disorders. The systemic form can involve essentially any organ but the diagnosis is most commonly made in the bone marrow. The current World Health Organization (WHO) classification of SM consists of histopathological criteria, tryptase level and immunophenotypic criteria by flow cytometry (Table I). Histopathological criteria include multifocal MCs in bone marrow (BM) or extracutaneous organs, abnormal MC morphology, and abnormal CD25 and/or CD2 expression (Jaffe *et al*, 2001; Valent *et al*, 2001).

The pathogenesis of SM appears to be related to altered tyrosine kinase (TK) activity. In particular, the receptor TK, kit, appears to play a central role. The activating *KIT* D816V mutation is characteristic of SM and is present in over 80% of cases regardless of disease classification. The majority of the remaining cases contain alternate *KIT* mutations (V560G, F522C, D820G among others; Gotlib, 2006). However, other alterations in TK activity may also be associated with SM, such

as the *FIP1L1-PDGFR* fusion seen in a subset of patients with SM and hypereosinophilia (Pardanani *et al*, 2003).

Detection of phosphoproteins is becoming possible with the development of phosphospecific antibodies. These can be used

Table I. WHO criteria for SM diagnosis.\*

Major criteria	
Multifocal dense infiltrates of MCs in bone marrow or other extracutaneous organ(s) (>15 MCs in aggregate) found in bone marrow sections (tryptase-stained)	
Minor criteria	
MCs in bone marrow or other extracutaneous organ(s) show an abnormal morphology, i.e. type I MCs (>25%) in bone marrow smears or in histological sections	
<i>KIT</i> mutation at codon 816 in extracutaneous organ(s)	
MCs in bone marrow express CD2 and/or CD25 (determined by flow cytometry)	
Serum total tryptase >20 ng/ml (does not count in patients who have associated haematological non-MC disorder)	

\*If at least one major and one minor, or at least three minor criteria are fulfilled, the diagnosis of SM can be established.  
MC, mast cell; SM, systemic mastocytosis.

to interrogate cells *in situ* for activation of signalling pathways. We have previously shown abnormal phospho-STAT5 (pSTAT5) patterns in BM from patients with non-chronic myeloid leukaemia (CML), chronic myeloproliferative disorders (CMPDs) harbouring the *JAK2* V617F, probably reflecting the increased TK activity of *JAK2* in this group of disorders (Szpurka *et al*, 2006; Aboudola *et al*, 2007). Given that STAT5 phosphorylation is also a downstream event of kit signalling (Gronney *et al*, 2005), we hypothesized that constitutive activation of kit leads to pSTAT5 accumulation in MC nuclei as determined by pSTAT5 immunohistochemistry (IHC). To this end, we studied a series of normal, CM and SM tissues to determine whether cases of SM could be identified through pSTAT5 expression pattern.

## Methods

### Patients

Archival tissue specimens were retrieved from the archives of the Division of Pathology and Laboratory Medicine using a computerized search for cases of SM and CM after obtaining Institutional Review Board approval. Non-neoplastic BM and other tissues with benign MCs were also retrieved for controls. One case of lymphoplasmacytic lymphoma (LPL) in BM was also retrieved as LPL is known to have increased reactive MCs. Specimens were fixed with B5 (BM), formalin (skin biopsies) or Hollande's fixative (one colon biopsy). Only samples fixed without delay were included, as phosphoprotein expression may decrease in resected tissue if there is a delay in fixation (Baker *et al*, 2005). These cases were then classified by a clinician with expertise in SM, based on WHO criteria, into one of the following categories after review of patient records for details regarding laboratory and pathological findings as well as clinical signs and symptoms, treatment and outcomes: CM, indolent SM, aggressive SM and SM-associated haematological non-MC disorder (Jaffe *et al*, 2001). Specifically, all 23 patients with systemic mast cell disease (SMCD; patients 10–32) had clinical evidence of SMCD. Patient 10 had MC aggregates in an extracutaneous organ (colon) verified by tryptase staining and also an abnormal phenotype (CD25<sup>+</sup>), and evidence of a *KIT* mutation. The remaining 22 patients had BM involvement by aggregates of tryptase + MCs. Fourteen had either elevated tryptase levels and/or *KIT* mutation, thus fulfilling criteria for SMCD. All had CD25 expression by

IHC as an added minor criterion needed for diagnosis of SMCD (Sotlar *et al*, 2004; Krokowski *et al*, 2005). For seven of the CM patients, the only clinical evidence of disease was skin, tryptase was not elevated when tested, and the age of these patients are fully consistent with CM rather than SMCD. The remaining two cases presented with skin lesions (Patients 8 and 9) but later developed haematological disorders for which they had BM biopsies; these will be discussed in detail in the *Results* section.

### DNA sequence analysis

Genomic DNA was extracted by using Qiagen DNeasy Tissue Kit or DNA Blood Kit (Qiagen, Valencia, CA, USA). PCR products amplifying both exon 11 and exon 17 of *KIT* were obtained by PCR. The PCR was performed in a final volume of 50 µl containing 3 mmol/l MgCl<sub>2</sub>, 0.4 µmol/l of each primer, 200 µmol/l of each deoxynucleotide triphosphate and U Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA, USA) by using an ABI 2720 cycler (Applied Biosystems, Foster City, CA, USA). After confirmation of PCR products by 1% agarose gel electrophoresis, PCR products were treated with ExoSAP (USB, Cleveland, OH, USA) and direct sequencing of purified PCR products was performed using an ABI 3730xl automated DNA sequencer using Dye Terminator DNA sequencing (Applied Biosystems). The sequences were aligned with the GenBank sequence of human *KIT* (accession number U63834).

### Immunohistochemistry

Immunohistochemistry was performed using an automated immunostainer (Benchmark, Ventana Medical Systems, Tucson, AZ, USA). Primary antibodies and pretreatments are listed in Table II. pSTAT5 staining and specificity of this antibody has been previously demonstrated (Aboudola *et al*, 2007). Briefly, Western blotting showed this antibody to blot a single band of appropriate size and was shown to be phosphospecific in cell lines manipulated with kinase inhibitors to decrease STAT5 phosphorylation (Aboudola *et al*, 2007). We also tested orthovanadate in staining buffers to inhibit tyrosine phosphatases that might be activated by antigen retrieval and diminish the amount of detectable phosphorylated proteins. We found no difference in staining quality between treated and non-treated BM control tissues

	Source	Dilution	Antigen retrieval
pSTAT5 (Y694/99)	Advantex BioReagents (Conroe, TX, USA)	1:500	CC1 (15 min, 95°C)
Tryptase	Dako (Carpinteria, CA, USA)	1:1000	Protease 1 (8 min)
CD2	Novocastra (Norwell, MA, USA)	1:10	CC1 (30 min, 95°C)
CD25	Novocastra (Norwell, MA, USA)	1:25	CC1 (30 min, 95°C)

Table II. Immunohistochemical primary antibodies.

CC1, proprietary high pH retrieval buffer (Ventana Medical Systems); Protease 1, proprietary protease reagent (Ventana Medical Systems).

(data not shown). Thus, tyrosine phosphatase inhibitors were not required for pSTAT5 IHC. Double immunostaining for tryptase and pSTAT5 was carried out in cases with scattered MCs using a sequential staining method with Ventana Red detection followed by standard diaminobenzidine (DAB; IVIEW, Ventana Medical Systems). All stains were scored without knowledge of the clinical diagnosis. pSTAT5-positive staining was defined as nuclear staining in more than 30% of MCs. The intensity of staining was categorized as strong or weak using erythroid precursors as an internal control for baseline staining in each case. Staining equal to or stronger than that of erythroid precursors was graded as 'strong'. Detectable staining that was less intense than neighbouring erythroid precursors was considered 'weak'. Samples without erythroid internal controls, such as skin and colon biopsies, were categorized as strong or weak based on the intensity of the positive control BM tissue section in each run.

Positive staining for CD2 and CD25 was defined by the presence of strong cytoplasmic and membranous staining, respectively, in >20% of MCs.

## Results

### *Clinical features of CM and SM patients*

Nine cases of CM were identified in the search of archival tissue specimens (Patients 1–9; Table III). Four patients were male; five female (Table III). Age ranged from <1 to 76 years old, but most patients were younger than 26 years of age. All of these patients presented with a rash which urticated upon stroking (Darier's sign) and the diagnosis was confirmed by skin biopsy. In four cases the lesion was completely excised; in one case an identical lesion elsewhere on the skin was not excised. In seven of the nine patients there were no additional clinical symptoms to suggest systemic involvement; in two of these seven cases a serum tryptase was checked, and was normal. None of these seven patients had a BM examination performed. In two of the nine cases (Patients 8 and 9) the patients were initially diagnosed with CM by skin biopsy and then had a BM biopsy performed for evaluation of haematological abnormalities (leucocytosis, anaemia and thrombocytopenia in Patient 8; leucocytosis and anaemia in Patient 9) years later. In both instances the patients were found to have a haematological malignancy – refractory anaemia with ringed-sideroblasts (Patient 8) and chronic myelomonocytic leukaemia (CMML; Patient 9). In both patients the BM examination did not demonstrate characteristic pathological features of SM. Patient 9 responded well to medical therapy and psoralen-ultraviolet A (PUVA) treatments; skin symptoms improved and serum tryptase fell with imatinib therapy. The skin symptoms returned when imatinib was discontinued because of side effects.

Nine patients with indolent systemic mastocytosis (ISM) were identified (Patients 10–18). Five patients were female; age ranged from 17 to 79 years old. All of the seven patients with

available clinical data presented with cutaneous manifestations; four of the seven patients also had gastrointestinal (GI) symptoms (Patients 10–12 and 15). All patients derived clinical benefit from the use of histamine antagonists to control symptoms; none of these patients had evidence of target organ damage and only Patient 18 was treated with cytoreductive therapy (dasatinib). Patients 16 and 17 presented with only skin lesions, had no other systemic complaints referable to mastocytosis, and had normal complete blood counts and metabolic profiles. However, both these patients had elevated serum tryptase >20 µg/l (21 µg/l in Patient 16 and 497 µg/l in Patient 17) and both patients' BM studies demonstrated morphological and IHC findings diagnostic of SM.

Six patients (Patients 19–24) were classified as having aggressive systemic mastocytosis (ASM), characterized by having target organ damage with evidence of organ impairment clinically assessed as likely from mastocytosis. Four patients were female; ages ranged from 47 to 78 years old. Serum tryptase ranged from 56 to 399 µg/l upon diagnosis. For many years, Patient 19 initially presented with skin lesions, which were lost upon development of systemic symptoms and organ involvement (hepatosplenomegaly, gastric varices, recurrent GI bleeding, pathological bone fractures). She had little response to hydroxycarbamide therapy and died 2.5 years after diagnosis. Patient 24 never presented with skin lesions, but had leucopenia, anaemia, hepatosplenomegaly, liver function abnormalities and recurrent episodes of anaphylaxis with hypotension. She responded clinically to interferon (IFN)- $\alpha$ 2b but had a history of coronary artery disease and suffered a ventricular tachycardia arrest. The four other patients with ASM (Patients 20–23) were found to have skin lesions upon presentation. All patients had symptomatic organ dysfunction of at least one organ. Patient 20 had hepatosplenomegaly with ascites controlled by a peritoneal shunt and declined IFN therapy; this patient expired from an acute myocardial infarction in the setting of progressive respiratory failure. Patient 21 was classified as having ASM, based on the presence of skin lesions, pathological vertebral fractures and BM criteria, but has not developed new bone or skin lesions and the serum tryptase has remained stable; he declined all cytoreductive therapy. Patient 22 was also classified as having ASM based on the presence of skin lesions, large bone lesions in the hip and BM criteria. Both ASM patients treated with imatinib (Patients 22 and 23) noted improved symptom control with reduction in serum tryptase.

Eight patients were identified to have SM (age range: 26–78 years) with an associated haematological non-MC disorder (SM-AH); no additional clinical information was available for one case (Patient 31). Of the seven classifiable patients, three had concomitant CMML (Patients 28–30). Patient 28 had mastocytosis skin lesions. There was little response to hydroxycarbamide but a transient clinical response to imatinib, which was discontinued because of side effects. Both Patients 29 and 30 described few symptoms referable to mastocytosis. Patient 29 presented with fatigue, cytopenias;

**Table III.** Clinical data on SM and CM.

Patient	Clinical Dx	Age (years)	Sex	S.tryptase ( $\mu\text{g/l}$ )*	Symptoms	Signs	Organ involvement	Treatment	Disease progression	Outcome
1	CM	19	F	NA	pr	Rash	Skin	None	No	Alive
2	CM	1	M	NA	pr	Rash	Skin	exc	No	Alive
3	CM	2	M	NA	pr	Rash	Skin	exc	No	Alive
4	CM	0.8	M	NA	pr	Rash	Skin	exc	One identical lesion	Alive
5	CM	26	M	NA	pr	Rash	Skin	exc	No	Alive
6	CM	1	F	8	pr	Rash	Skin	exc	No	Alive
7	CM	6	F	8	pr, fl	Rash	Skin	AH	Persistent pr, flushing	Alive
8	CM (also MDS)	65	F	NA	pr, fa	Rash, anaemia, Lcytosis, Tcytopenia	Skin	AH	Progressive skin involvement	Alive
9	CM (also CMML)	76	F	160	pr, fa	Rash, anaemia, Lcytosis	Skin	AH, PUVA, imatinib	Progressive skin involvement	Dead 4.5 years after dx
10	ISM	17	F	323	pr, fl, abd. P, V, D	Rash	Skin; GI	AH, H2A, st	Stable	Alive
11	ISM	32	M	200	pr, fl, abd. P, D, back P	Rash	Skin; GI	AH, H2A	Stable	Alive
12	ISM	79	F	200	fl, abd. P, D, WL	Rash; splenomegaly	Skin; GI	AH, H2A, gastr	Stable	Alive
13	ISM	46	M	NA	NA	NA	NA	NA	NA	NA
14	ISM	46	M	NA	NA	NA	NA	NA	NA	NA
15	ISM	49	F	NA	Pr, fl, epigastric P	Rash	Skin; GI	AH, H2A	Stable	Alive
16	ISM	36	M	21	pr	Rash	Skin	AH, H2A	Stable	Alive
17	ISM	76	F	497	pr	Rash	Skin	AH, H2A	Stable	Alive
18	ISM	64	F	41	pr, fa	Rash	Skin	AH, PUVA, dasatinib	Progressive skin involvement	Alive
19	ASM	78	F	168	abd. P, D, GI bl, fa, WL, bone P	Hsmeg, varices, path. fx, anaemia, transf. dep.	Skin; GI; bone; haem	AH, H2A, st, gastr, hc	Progressive symptoms	Dead 2.5 years after dx
20	ASM	65	F	399	sync, hypot, pr, ascites, D, fa, WL	Rash, hsmeg, Lcytosis, anaemia	Skin; GI; haem	AH, H2A, st, gastr	Progressive symptoms	Dead 5 years after dx
21	ASM	63	M	88	pr, back P	Rash, path. Fx	Skin; bone	AH, H2A	Stable	Alive
22	ASM	47	M	56	pr	Rash, bone lesions	Skin; bone	AH, H2A, imatinib; PUVA; skin LT	Transient improvement	Alive
23	ASM	49	F	139	pr, abd. P, D, hypot, fa	Rash, anaemia	Skin; GI; haem	AH, H2A, st, gastr; imatinib	Transient symptom improvement	Alive
24	ASM	57	F	200	hypot, sync, fl, wheezing, N, V, abd. P	Lpenia, anaemia, hsmeg, elevated LFTs	haem; GI	AH, H2A, st, gastr; IFN- $\alpha$	Transient symptom improvement	Dead 1 year after dx
25	SM-AH (HL)	52	F	NA	pr, bone P, dyspnoea	Rash, path. fx, pleural effusion	Skin; bone	allo BMT, AH, H2A, st	Stable	Alive
26	SM-AH (AML)	78	M	NA	fa, dizziness	Lpenia	Haem	Cytarabine and mitoxantrone	Relapsed AML	Dead 1.5 years after dx
27	SM-AH (AML)	51	M	NA	fa, dyspnoea, anorexia	Lpenia, anaemia, Tcytopenia	Haem	allo BMT, st, ciclosporin	GVHD, liver failure	Dead 4 months after dx
28	SM-AH (CMML)	78	M	95	fa, rash, pr, bl, abd. pain	Tcytopenia, anaemia, hsmeg	Skin; GI; haem	AH, H2A, st, gastr, hc, imatinib	Transient symptom improvement	Dead 2 years after dx

Table III. (Continued).

Patient	Clinical Dx	Age (years)	Sex	S.tryptase ( $\mu\text{g/l}$ )*	Symptoms	Signs	Organ involvement	Treatment	Disease progression	Outcome
29	SM-AH (CMML)	73	F	199	Syncopal, hypot, GI bl, abd. P, fa	Anaemia, Lcytosis, Tcytopenia	haem; GI	AH, H2A, st, gastr, thalidomide	Progressive symptoms	Dead 5 months after dx
30	SM-AH (CMML)	75	M	324	fa, abd. P, anorexia	Anaemia, Lcytosis, hsmeg	haem	AH, H2A, st, IFN- $\alpha$	Transient symptom improvement	Alive
31	SM-AH	47	F	NA	NA	NA	NA	NA	NA	NA
32	SM-HES	26	M	44	None	Peripheral eosinophilia	Haem	Imatinib	Remission	Alive

Dx, diagnosis; S.tryptase, serum tryptase; CM, cutaneous mastocytosis; CMML, chronic myelomonocytic leukaemia; MDS, myelodysplastic syndrome; ISM, indolent systemic mastocytosis; ASM, aggressive systemic mastocytosis; SM-AH, systemic mastocytosis with an associated haematological non-mast cell disorder; HL, Hodgkin lymphoma; AML, acute myeloid leukaemia; HES, hypereosinophilic syndrome; F, female; M, male; NA, not available.

Symptoms: pr, pruritis; fl, flushing; fa, fatigue; abd., abdominal; P, pain; V, vomiting; D, diarrhoea; WL, weight loss; GI, gastrointestinal; bl, bleeding; sync, syncopal; hypot, hypotension; N, nausea.

Signs: Lcytosis, leucocytosis; Tcytopenia, thrombocytopenia; Hsmeg, hepatosplenomegaly; haem, haematological (bone marrow); path. fx, pathological fractures; transf. dep., transfusion-dependent; Lpenia, leucopenia; LFTs, liver function tests.

Treatment: exc, excision; AH, antihistamines; imat, imatinib; H2A, H<sub>2</sub> antagonists; st, steroids; gastr, Gastrocom; hc, hydroxycarbamide; LT, laser therapy; IFN, interferon; allo BMT, allogeneic bone marrow transplant.

\*Reference range 1.9–13.5  $\mu\text{g/l}$ .

and the BM demonstrated CMML with concomitant mastocytosis. The patient declined IFN therapy and was treated with thalidomide; the patient expired from renal failure and respiratory failure. Patient 30 also presented with fatigue and was found to have anaemia and leucocytosis with a BM examination demonstrating CMML with concomitant mastocytosis. This patient responded to IFN- $\alpha$  and is currently alive. Two patients had acute myeloid leukaemia (AML; Patients 26 and 27), one had Hodgkin lymphoma (HL) with subsequent myelodysplastic syndrome and SM (Patient 25) and one had hypereosinophilic syndrome (HES; Patient 32). Patients 25 (HL) and 27 (AML) were treated with an allogeneic BMT. Patient 25 remains in remission from HL but the post-transplant BM demonstrated SM. Patient 27 expired from liver failure 3 months after the transplant. Patient 26 (AML) was treated with cytarabine and mitoxantrone and expired from relapsed AML 1.5 years after diagnosis; SM was diagnosed unexpectedly in the BM, as the patient had no cutaneous or systemic symptoms referable to mastocytosis. Patient 32 was found to have a peripheral eosinophilia of 65% (absolute eosinophil count of 13 420) and subsequent evaluation demonstrated *FIP1L1-PDGFR $\alpha$*  + myeloproliferative variant HES. He was otherwise asymptomatic. The patient is currently in remission on imatinib therapy.

#### Normal mast cells and mast cells in most cases of cutaneous mast cell disease lack nuclear pSTAT5

Dual colour immunohistochemical staining (tryptase red, pSTAT5 brown) was performed to determine the pSTAT5 expression pattern in reactive MCs. Examination of eight

BMs that were negative for malignancy (marrows performed for lymphoma staging, thrombocytopenia or anaemia) showed that non-neoplastic MCs failed to accumulate detectable nuclear pSTAT5 (Fig 1). An additional BM involved by LPL was also studied. LPLs are known to contain an increase in reactive MCs and this was also negative. We also examined skin (two), leiomyomas (two), lung adenocarcinomas (two), and these also showed MCs negative for pSTAT5.

Nine cases of CM were studied and eight showed no detectable nuclear pSTAT5 in MCs. Interestingly, Patient 8, a 65-year-old female with maculopapular CM, showed

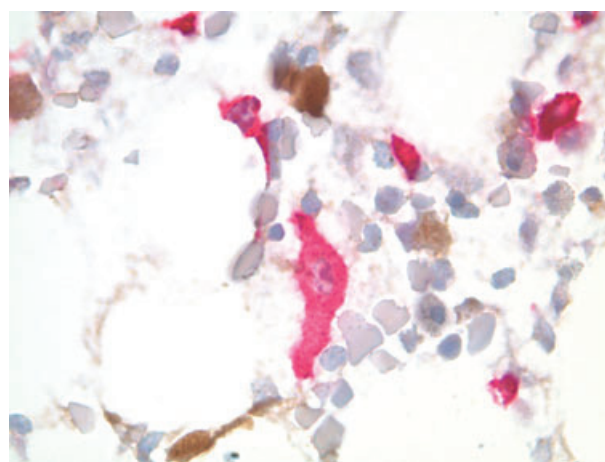


Fig 1. Absence of nuclear pSTAT5 (brown) in non-neoplastic mast cells from a bone marrow without systemic mastocytosis. Mast cells are highlighted with tryptase in red but lack pSTAT5 (1000 $\times$ ).

Table IV. Laboratory data on SM and CM.

Patient	Tissue	Clinical diagnosis	KIT mutation	IHC			pSTAT5 IHC		Intensity
				Tryptase	CD2	CD25	Pos.	Pos. MCs (%)	
1	Skin	CM	NA	+	-	-	-	0	
2	Skin	CM	NA	+	-	-	-	<10	S
3	Skin	CM	NA	+	-	-	-	<10	W
4	Skin	CM	NA	+	-	-	-	<10	S
5	Skin	CM	NA	+	-	-	-	<10	W
6	Skin	CM	NA	+	-	-	-	0	
7	Skin	CM	NA	+	-	-	-	<10	S
8	Skin	CM	NA	+	Weak	+	+	75	S
9	Skin	CM	NA	+	-	-	-	0	
10	Colon	ISM	D816V*	+	-	+	+	50	W
11	BM	ISM	wt*	+	-	+	+	75	S
12	BM	ISM	wt*	+	-	+	+	75	S
13	BM	ISM	NA	+	-	+	+	50	S
14	BM	ISM	NA	+	-	+	+	30	W
15	BM	ISM	NA	+	-	+	+	50	S
16	BM	ISM	wt*	+	-	+	+	75	S
17	BM	ISM	D816V***	+	-	+	+	90	S
18	BM	ISM	NA	+	-	+	+	90	S
19	BM	ASM	NA	+	-	+	+	90	S
20	BM	ASM	D816V**	+	-	+	+	50	S
21	BM	ASM	wt*	+	-	+	+	50	S
22	BM	ASM	wt*	+	-	+	+	75	S
23	BM	ASM	wt*	+	-	+	+	75	S
24	BM	ASM	D816Y**	+	-	+	+	30	W
25	BM	SM-AH	NA	+	-	+	+	50	S
26	BM	SM-AH	NA	+	-	+	+	50	S
27	BM	SM-AH	NA	+	-	+	+	75	S
28	BM	SM-AH	D816V**	+	-	+	+	75	S
29	BM	SM-AH	D816V**	+	-	+	+	75	S
30	BM	SM-AH	D816V***	+	-	+	+	50	S
31	BM	SM-AH	NA	+	-	+	+	90	S
32	BM	SM-HES	NA	+	-	+	+	90	S

\*Peripheral blood; \*\*bone marrow; \*\*\*both.

BM, bone marrow; F, female; M, male; CM, cutaneous mastocytosis; ISM, indolent systemic mastocytosis; ASM, aggressive systemic mastocytosis; SM-AH, systemic mastocytosis with an associated haematological non-mast cell disorder; NA, not available; wt, wild type; Pos., positive; S, strong; W, weak; HES, hypereosinophilic syndrome.

accumulation of pSTAT5 in MCs (Table IV). In addition to nuclear pSTAT5 expression, her skin biopsy showed large aggregates of MCs with weak CD2 cytoplasmic, and CD25 cytoplasmic and membranous staining. A BM biopsy showed no evidence of SM. Unfortunately, molecular studies were not available on this patient and thus we cannot exclude the possibility of *KIT* mutation in this case of CM (Buttner *et al*, 1998; Longley *et al*, 1999; Sotlar *et al*, 2003).

#### Mast cells from SM express nuclear pSTAT5

Twenty-three patients with SM had available tissue for immunohistochemical analysis. They represented a spectrum of clinical disease including nine ISM, six ASM, seven SM with associated haematological disorders and one SM with

eosinophilic leukaemia (*FIP1L1-PDGFR*<sup>+</sup>; Tables III and IV). MC pSTAT5 was detected in all SM cases (100%). This was seen in the nuclei of the majority of MCs (mean 65%, range: 30–90; Fig 2). As seen previously in BM, occasional erythroid precursors (approximately 10%, often in small clusters) and maturing granulocytes (approximately 10%) had detectable nuclear pSTAT5 (Aboudola *et al*, 2007), while occasional megakaryocytes had cytoplasmic staining but lacked detectable nuclear pSTAT5.

Interestingly, one case of SM (Patient 32) included a case of SM with hypereosinophilic syndrome (SM-HES) harbouring the *FIP1L1-PDGFR* as detected by *CHIC2* deletion by FISH. In this disorder, patients have a marked eosinophilia, elevated serum tryptase levels (as was present in this patient) and increased interstitially distributed spindle MCs in BM

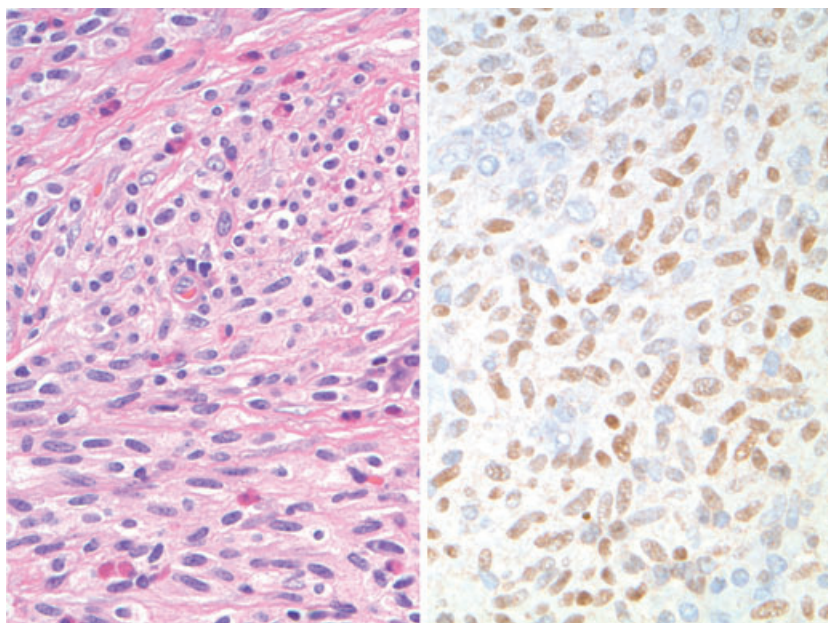


Fig 2. Systemic mastocytosis bone marrow with diffuse nuclear pSTAT5 staining (Patient 25; 400 $\times$ ).

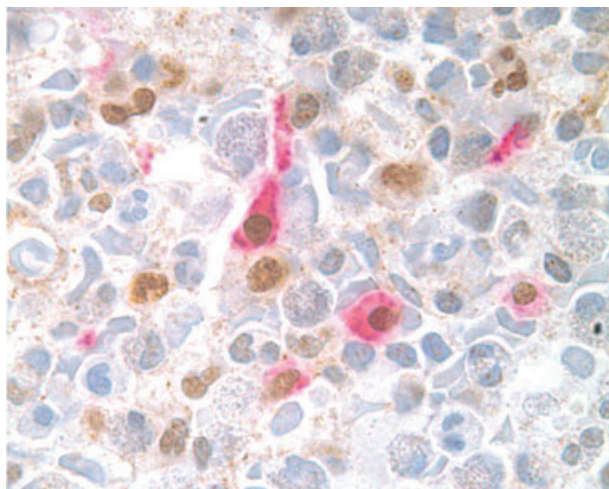


Fig 3. Systemic mastocytosis-hypereosinophilic syndrome mast cells (MCs) have detectable nuclear pSTAT5 as demonstrated with dual immunolabelling for tryptase (red) and pSTAT5 (brown; Patient 32). Note nuclear pSTAT5 in MCs (1000 $\times$ ). A few non-MCs (granulocytic cells) also appear to express nuclear pSTAT5.

without clear MC lesions/clusters. Double immunostaining with tryptase and pSTAT5 showed accumulation of nuclear pSTAT5 in MCs (Fig 3), which also had membranous expression of CD25.

Of the 23 samples evaluated, 22 were BM biopsies and one was a colon biopsy diffusely involved by MCs. The colon biopsy was obtained from a patient with ISM (Patient 10) that had detectable nuclear pSTAT5 in 50% of MCs. The MCs also expressed CD25, and peripheral blood leucocytes harboured the D816V *KIT* mutation.

Aside from nuclear pSTAT5 detection in BM MCs, we found expression of pSTAT5 in nuclei of MCs from the colon of a patient with ISM, as well as in MC nuclei from skin biopsies of patients with SM (Patients 15, 17, 20 and 25). pSTAT5 may thus be a useful marker of neoplastic MCs in sites other than BM.

#### *Correlation with molecular and immunophenotypic data*

We assessed both CD25 and CD2 by IHC, markers of abnormal MCs when studied by flow cytometry (CD2 and CD25) or IHC (CD25) (Escribano *et al*, 2002; Sotlar *et al*, 2004). We found CD25 to be a robust marker in BM trephine section IHC. 23/23 cases of SM expressed CD25 and were the same cases in which pSTAT5 could be demonstrated. CD2 was insensitive, despite adequate internal T-cell controls (Fig 4).

Molecular genetic data on *KIT* D816V status was available in 13 SM patients. Six patients were heterozygous for the D816V mutation, one patient had a D816Y mutation and the remaining six were wild type at exons 11 and 17. The six 'wild-type' patients had no detectable mutation in peripheral blood leucocytes, and unfortunately had no BM available for molecular testing (Table IV).

#### **Discussion**

It is now known that >80% of cases of SM harbour a mutation in the ATP-binding domain of the receptor TK kit that results in a gain of function (Longley *et al*, 1996; Valent *et al*, 2005; Garcia-Montero *et al*, 2006). Activation of kit causes downstream signalling events involving several pathways (Deberry *et al*, 1997; Linnekin, 1999; Wang *et al*, 2000; Reber *et al*,

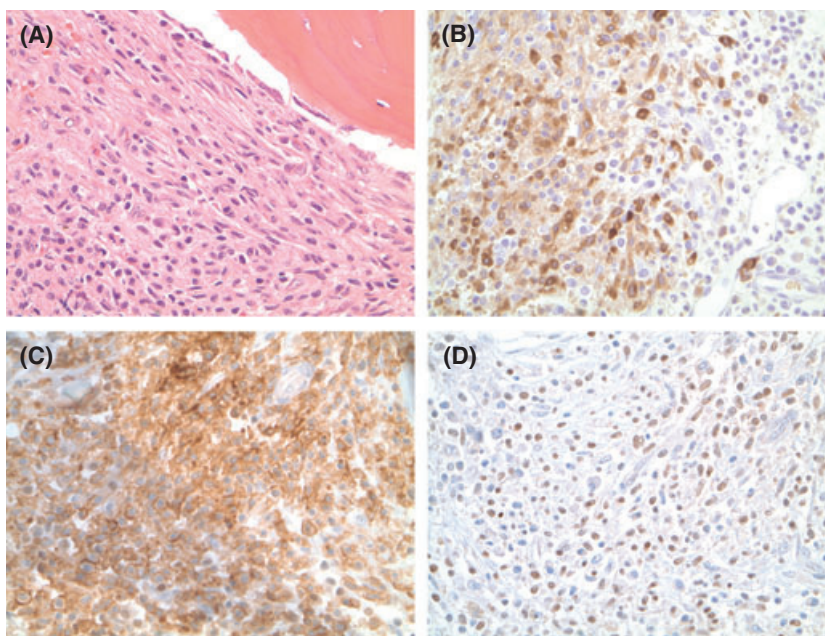


Fig 4. Systemic mastocytosis bone marrow with sheets of mast cells (MCs; A, haematoxylin and eosin). MCs are positive for tryptase (B), CD25 (C) and nuclear pSTAT5 (D; Patient 20, 400 $\times$ ).

2006) that, among other effects, results in phosphorylation of STAT5 (Brizzi *et al*, 1999). We hypothesized that this might be detectable by IHC in MCs, thus reflecting *in situ* activation of STAT5 because of abnormal kit activation. In addition, detection of pSTAT5 might be a useful diagnostic marker for distinguishing normal from neoplastic MCs (Ogata *et al*, 1997, 1998).

We found that 100% of SM expressed abnormal nuclear pSTAT5 in BM MCs, while most CM and all non-neoplastic MCs lacked pSTAT5. Interestingly, biopsies from cutaneous lesions and an involved GI biopsy from patients with SM also contained pSTAT5 + MCs. Thus, nuclear pSTAT5 expression is a fairly specific marker of neoplastic Mcs, not only in the BM, but also at other anatomic sites. Furthermore, abnormal pSTAT5 expression was also detected in a case of SM with eosinophilia with a *FIP1L1-PDGFR $\alpha$*  fusion. Thus, nuclear pSTAT5 in MCs appears to be a relatively robust abnormal phenotypic marker of SM.

Historically, the incidence of detecting the mutation has been quite variable, presumably due to technical variations in assays. Recent data suggest that the majority (80%) of neoplastic MC contain this mutation and other cases may harbour rare mutations at other sites (Pardani, 2005; Valent *et al*, 2005; Garcia-Montero *et al*, 2006; Gotlib, 2006). Unfortunately, we were only able to obtain molecular studies in a subset of cases. Our relatively low rate of *KIT* D816V detection (46%) may be related to technical issues of detection sensitivity, given the lower frequency of MC progenitor cells harbouring *KIT* D816V in peripheral blood. The incidence of the *KIT* mutation identified in the peripheral blood ranges from 22% to 78% depending upon the cell populations studied

in the peripheral blood and the method of analysis (Worobec *et al*, 1998; Akin *et al*, 2000).

Given the variability in ability to detect this abnormality by amplification methods, the detection of pSTAT5 by IHC may be a reliable surrogate test. Furthermore, it appears that pSTAT5 is abnormally activated in *KIT* mutations other than D816V. Indeed, we detected pSTAT5 in the one patient with D816Y mutation and in the patient with *FIP1L1-PDGFR $\alpha$* . Thus, pSTAT5 IHC is a marker of abnormal MCs.

We have previously performed pSTAT5 IHC in BMs of patients with *JAK2* V617F and found abnormal nuclear megakaryocytic pSTAT5 staining on such cases (Aboudola *et al*, 2007). Because the *KIT* mutation in SM appears to involve early haematopoietic progenitor cells (Akin *et al*, 2000), we considered the possibility at the onset of this study that a similar pattern might be seen or that erythroid or granulocytic cells would show increased immunoreactivity. However, this was not the case. We observed no increase in pSTAT5 in maturing haematopoietic cells within the BM. This implies that while the *KIT* mutation may be present in the genomic DNA of a subset of haematopoietic cells, it is primarily in MCs where the kit protein is expressed and the signalling pathway is active (Akin *et al*, 2000).

Currently, abnormal expression of CD2 and CD25 by flow cytometry are considered minor criteria useful in the diagnosis of SM (Jaffe *et al*, 2001; Escribano *et al*, 2002; Pardani, 2005). In comparison to CD2 and CD25, pSTAT5 was superior to CD2 immunohistochemical staining in BM for detection of abnormal MCs. We found CD2 IHC to be insensitive, as others have also reported (Krokowski *et al*, 2005). CD25 was seen in a similar proportion of cases as pSTAT5 by IHC. pSTAT5 has

the advantage of being expressed in a nuclear pattern. Thus, it is amenable to double labelling in bright field applications with tryptase as illustrated in the *FIP1L1-PDGFR $\alpha$*  case, in which MCs were difficult to identify as they are present interstitially without large clusters. Because tryptase stains cells in a cytoplasmic pattern, the nuclear expression of pSTAT5 is easily recognizable in dual-labelled cells. Immunohistochemistry in archival tissue also has the advantage of retrospective analysis and does not require one to analyse samples in 24–48 h as in flow cytometry. This is particularly important because some cases of SMCD may not be clinically suspected and special studies for MC phenotype by flow cytometry are not routinely performed. Thus, if not suspected initially, a flow cytometry sample may not be suitable for subsequent analysis for SMCD.

Although the functional significance of CD2 and CD25 expression by neoplastic MC is still unclear, there is emerging evidence that STAT5 plays an important role in MC biology. *STAT5A/B* gene-deleted mice are reported to be devoid of MCs, a phenotype similar to the naturally occurring *W/W<sup>v</sup>* and *Sl/Sl<sup>d</sup>* mice that have mutations in the *KIT* and stem cell factor genes respectively (Shelburne *et al.*, 2003). In addition to providing survival and developmental signals, STAT5 activation may also be critical for MC function with regard to degranulation and cytokine production (Barnstein *et al.*, 2006). Thus, identification of constitutive pSTAT5 in neoplastic MCs appears to be a mechanism-based diagnostic tool directly related to the pathogenesis of SM.

In conclusion, we studied a series of SM and normal tissues and demonstrated that activated STAT5 (nuclear pSTAT5) is present in MCs in the vast majority of SM cases while normal MCs lack detectable pSTAT5. This 'functional' IHC application has diagnostic utility. It compares favourably with other phenotypic abnormalities in SM (CD25 expression). Because of the nuclear localization, it is amenable to dual colour IHC with cytoplasmic markers of MCs such as tryptase, making it useful in cases where large clusters of MCs are not easily identifiable for assessment of pSTAT5 or CD25. We propose consideration of pSTAT5 expression by IHC as a new minor diagnostic criterion for the diagnosis of SM in future classification systems.

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