

Gene expression profile of highly purified bone marrow mast cells in systemic mastocytosis

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Background: Despite the fact that a great majority (>90%) of patients with systemic mastocytosis (SM) carry a common genetic lesion, the D816V *KIT* mutation, little is known regarding the molecular and biological pathways underlying the clinical heterogeneity of the disease.

Objective: We sought to analyze the gene expression profile (GEP) of bone marrow mast cells (BMMCs) in patients with SM and its association with distinct clinical variants of the disease.

Methods: GEP analyses were performed by using DNA-oligonucleotide microarrays in highly purified BMMCs from patients with SM carrying the D816V *KIT* mutation (n = 26) classified according to the diagnostic subtype of SM versus normal/reactive BMMCs (n = 7). Validation of GEP results was performed with flow cytometry in the same set of samples and in an independent cohort of 176 subjects.

Results: Overall, 758 transcripts were significantly deregulated in patients with SM, with a common GEP (n = 398 genes) for all

subvariants of SM analyzed. These were characterized by upregulation of genes involved in the innate and inflammatory immune response, including interferon-induced genes and genes involved in cellular responses to viral antigens, together with complement inhibitory molecules and genes involved in lipid metabolism and protein processing. Interestingly, aggressive SM additionally showed deregulation of apoptosis and cell cycle-related genes, whereas patients with indolent SM displayed increased expression of adhesion-related molecules.

Conclusion: BMMCs from patients with different clinical subtypes of SM display distinct GEPs, which might reflect new targetable pathways involved in the pathogenesis of the disease. (J Allergy Clin Immunol 2013;■■■■:■■■■-■■■■.)

Key words: Systemic mastocytosis, D816V *KIT* mutation, gene expression profile, mast cells, cDNA microarrays

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Systemic mastocytosis (SM) comprises a heterogeneous group of disorders characterized by abnormal growth and accumulation of clonal mast cells (MCs) in 1 or more organ systems.^{1,2} Based on their clinicopathologic features, 7 variants of SM are currently recognized by the World Health Organization (WHO), including mild (eg, indolent systemic mastocytosis [ISM]) and aggressive (eg, aggressive systemic mastocytosis [ASM] and mast cell leukemia) forms of the disease.^{1,2} In addition, recent studies point out the existence of 2 additional subvariants of ISM, well-differentiated systemic mastocytosis and ISM without skin lesion (ISMs—), with unique clinicobiological and immunophenotypic features and different therapeutic requirements.³⁻⁸ Despite the fact that the distinct subtypes of SM are clinically well-defined entities, little is known about the molecular mechanisms, biological functions, and pathways underlying disease heterogeneity because the great majority (>90%) of these patients (except those with well-differentiated systemic mastocytosis) carry a common genetic lesion, the stem cell growth factor receptor gene (*KIT*) D816V activating mutation.⁹

Gene expression profiling with cDNA microarray technology has largely contributed to a better understanding of the molecular mechanisms underlying normal and altered biological processes in many disease conditions.^{10,11} However, reports about the cDNA microarray analysis of gene expression profiles (GEPs) of SM are scarce, and they have either considered SM as a single entity¹² or focused on single disease subvariants (eg, ISM).^{13,14} Most importantly, in such studies either bone marrow (BM) mononuclear cells¹² or whole peripheral blood samples^{13,14} containing variable (typically low) percentages of MCs were used to assess GEPs; indeed, none of these studies specifically investigated the GEPs of highly purified bone marrow mast cell

Abbreviations used

AHNMD: Associated clonal hematologic non-mast cell lineage disease
 ASM: Aggressive systemic mastocytosis
 BM: Bone marrow
 BMMC: Bone marrow mast cell
 GEP: Gene expression profile
 ISM: Indolent systemic mastocytosis
 ISMs-: Indolent systemic mastocytosis without skin lesions
 ISMs+: Indolent systemic mastocytosis with skin lesions
 MC: Mast cell
 PAM: Prediction Analysis for Microarrays
 PI: Proliferation index
 ROC: Receiver operating characteristic
 SM: Systemic mastocytosis
 SSM: Smoldering systemic mastocytosis
 STAT: Signal transducer and activator of transcription
 WHO: World Health Organization

(BMMC) fractions. Consequently, such studies provide biased and complex results that are difficult to interpret depending on the degree of BM infiltration by MCs and the variable distribution within individual samples of other different BM and peripheral blood cell populations.^{15,16}

Here, for the first time, we analyze the GEPs of highly purified BMMCs from patients with ISM and patients with ASM who carried in common the D816V *KIT* mutation versus normal/reactive BMMCs. Overall, our results show a common GEP for ISM and ASM, together with GEPs specific for each of these 2 major subtypes of the disease.

METHODS**Patients**

Only adult (≥ 18 years) patients who provided prior informed consent, met the WHO criteria for SM,^{1,2} and carried the *KIT* D816V mutation were included in this study. Overall, BM aspirates were obtained from 139 patients (76 male and 63 female patients; median age, 52 years; age range, 23–82 years) given a diagnosis of SM at the reference centers of the Spanish Network on Mastocytosis (Hospital Virgen del Valle, Toledo, Spain, and the Cancer Research Centre, Salamanca, Spain).^{1,2} Patients were classified according to the WHO criteria^{1,2} and more recent recommendations^{4–6} as 35 cases of ISM without skin lesions (ISM_s-), 80 cases of ISM with skin lesions (ISM_s+; including 2 cases of smoldering systemic mastocytosis [SSM]), 14 cases of ASM, and 10 cases of SM-associated clonal hematologic non-mast cell lineage disease (AHNMD; 4 cases of ISM-AHNMD and 6 cases of ASM-AHNMD). For the purpose of this study, patients with ISM-AHNMD and patients with ASM-AHNMD were grouped together with patients with ISM and patients with ASM, respectively. Additionally, control BM aspirates were obtained from 70 subjects (37 male and 33 female subjects; median age, 59 years; age range, 23–89 years), including 9 healthy donors (normal BM) and 61 patients undergoing BM aspiration for clinical reasons other than mastocytosis or a myeloid neoplasm. In all cases informed consent was obtained according to the guidelines of the local ethics committees of the 2 centers.

Isolation of MCs and other subpopulations of BM nucleated cells, DNA extraction, and detection of the D816V *KIT* mutation

Isolation of MCs and other subpopulations of BM cells was performed as previously described in detail (see the Methods section in this article's Online Repository at www.jacionline.org).^{9,17}

RNA extraction, microarray hybridization, and data analysis

Assessment of GEPs was performed on RNA extracted from purified BMMCs from 33 subjects from whom enough mRNA was obtained for the GEP hybridization protocols. In detail, GEP studies were performed in 7 control subjects (5 healthy donors and 2 subjects carrying a B-cell lymphoproliferative disorder who exhibited reactive MC hyperplasia [cases 6 and 7]) and 26 patients with SM (Table 1) by using the Human Gene 1.0 ST Array (Affymetrix, Santa Clara, Calif; see the Methods section in this article's Online Repository).

For data analysis, GEP raw data (Affymetrix.CEL files) were normalized by using the Robust Multi-array Average (RMA) algorithm, which included background correction, quantile normalization, $\log_2$ transformation, and probe set summarization.¹⁸ Gene selection criteria used to decrease the risk for false-positive results are described in detail in the Methods section in this article's Online Repository. Differentially expressed genes between normal/reactive samples and each diagnostic subtype of SM were identified by using supervised 2-class unpaired Significance Analysis of Microarray (SAM),¹⁹ as assessed with MultiExperiment Viewer version 4.6.1 software²⁰ based on a combined cutoff for a false discovery rate of less than 0.01 and an absolute fold change of 2.0 or greater.

For gene ontology term annotation and functional enrichment analysis of significantly deregulated genes, the GeneCodis (Gene Annotation Co-occurrence Discovery)²¹ functional annotation tool was used. Analysis of gene interactions and correlation networks was performed by using the Ingenuity Pathway Analysis software (Ingenuity Systems, www.ingenuity.com).

For the identification of the best combination of genes that would allow for discrimination between patients with ISM and those with ASM, the Prediction Analysis for Microarrays (PAM) software (version 2.1; University of Stanford, Stanford, Calif) based on a nearest shrunken centroid algorithm was used to build a classifier based on those genes significantly deregulated in patients with ISM, patients with ASM, or both ($n = 758$),²² as described in detail in the Methods section in this article's Online Repository.

Multiparameter flow cytometric immunophenotypic studies and cell-cycle analyses

Multiparameter flow cytometric immunophenotypic studies were used to validate microarray results for a set of 10 genes (*CD2*, *CD4*, *CD33*, *CD52*, complement receptor 2 [*CR2*], low affinity immunoglobulin gamma Fc region receptor II-b [*FCGR2B*], major histocompatibility complex class II, DR beta 5 chain [*HLA-DRB5*], interleukin 2 receptor alpha chain [*IL2RA*], interleukin 3 receptor alpha chain [*IL3RA*], and leukocyte-associated immunoglobulin-like receptor 1 [*LAI1*]; see Table E1 in this article's Online Repository at www.jacionline.org) that code for proteins primarily located at the cell-surface membrane and that have not previously been described to be stored in BMMC granules, none of which were used here during sorting experiments. For these studies, BM aspirates from the same patients initially included in the microarray analyses (validation set 1, Table 1) plus another independent group of patients ($n = 176$, validation set 2) were processed as previously described,⁸ according to the recommendations of the Spanish Network on Mastocytosis.²³

Assessment of the cell-cycle distribution of BMMCs (percentage of G₀/G₁ and S plus G₂/M phases) was performed by using a previously described 3-color flow cytometric technique (see the Methods section in this article's Online Repository).²⁴

RESULTS**Overall transcription profile of BMMCs in patients with SM**

Supervised analysis of the GEPs of BMMCs from patients with SM revealed a distinct pattern between patients with ISM and those with ASM but with a common imprint for a total of 758 deregulated transcripts versus normal/reactive BMMCs; upregulated expression of *KIT* and tryptase alpha/beta 1 (*TPSAB1*) was found at similar

TABLE I. Clinical and biological characteristics of adult patients with SM (n = 26) and control subjects (n = 7) in which the GEPs of BMMCs were analyzed by using cDNA microarrays

Case ID	Diagnostic group	Sex (M/F)	Age (y)	Percentage of BMMCs by flow cytometry	Skin lesions	Hepatomegaly	Splenomegaly	Serum tryptase (ng/mL)	Pattern of involvement of BM cells by the <i>KIT</i> D816V mutation*
1	Control BM	F	42	0.046	No	NA	NA	NA	Nonmutated
2	Control BM	M	41	0.003	No	NA	NA	NA	Nonmutated
3	Control BM	F	71	0.006	No	NA	NA	NA	Nonmutated
4	Control BM	M	26	0.002	No	NA	NA	NA	Nonmutated
5	Control BM	M	62	0.02	No	NA	NA	NA	Nonmutated
6	Control BM	F	59	0.06	No	NA	NA	NA	Nonmutated
7	Control BM	M	71	0.21	No	NA	NA	NA	Nonmutated
8	ISMs—	F	63	0.29	No	No	No	20.5	Restricted to MCs
9	ISMs—	F	28	0.12	No	No	No	51.4	Restricted to MCs
10	ISMs—	M	58	0.21	No	Yes	No	38.1	Restricted to MCs
11	ISMs+	M	46	1.0	Yes	No	No	NA	Restricted to MCs
12	ISMs+	F	54	0.52	Yes	No	No	60.4	Restricted to MCs
13	ISMs+	M	24	0.89	Yes	No	No	65.2	Restricted to MCs
14	ISM-AHNMD†	M	61	0.13	No	Yes	Yes	44.5	Restricted to MCs
15	ISMs+	M	51	0.43	Yes	No	No	98.1	Myeloid lineages
16	ISMs+	M	32	0.31	Yes	No	No	34.9	Myeloid lineages
17	ISMs+	M	31	0.52	Yes	Yes	No	115.0	Myeloid lineages
18	ISMs+ (SSM)	M	61	4.9	Yes	Yes	Yes	225.0	Myeloid lineages
19	ISMs+ (SSM)	M	62	2.8	Yes	Yes	Yes	1500.0	Myeloid + lymphoid lineages
20	ISMs+	F	47	0.39	Yes	No	No	72.6	Myeloid + lymphoid lineages
21	ISMs+	F	64	0.28	Yes	No	Yes	22.4	Myeloid + lymphoid lineages
22	ASM-AHNMD‡	M	64	0.13	No	No	Yes	NA	Myeloid lineages
23	ASM	M	46	2.6	Yes	Yes	Yes	370.0	Myeloid lineages
24	ASM	M	72	1.8	No	No	Yes	NA	Myeloid lineages
25	ASM	F	76	18.0	Yes	Yes	Yes	302.0	Myeloid lineages
26	ASM	M	64	2.9	Yes	Yes	Yes	1625.0	Myeloid lineages
27	ASM	M	69	0.13	Yes	Yes	Yes	52.6	Myeloid lineages
28	ASM-AHNMD§	F	55	18.0	No	Yes	Yes	NA	Myeloid lineages
29	ASM	M	41	1.3	Yes	Yes	Yes	404.0	Myeloid + lymphoid lineages
30	ASM	M	75	0.86	No	No	Yes	85.0	Myeloid + lymphoid lineages
31	ASM	M	58	1.9	No	Yes	Yes	184.0	Myeloid + lymphoid lineages
32	ASM	M	67	0.08	No	No	No	NA	Myeloid + lymphoid lineages
33	ASM-AHNMD	M	82	5.3	No	Yes	Yes	>200.0	Myeloid + lymphoid lineages

F, Female; M, male; NA, no available data.

*The following BM cell lineages other than MCs were analyzed: (1) CD34⁺ hematopoietic precursors; (2) lymphoid cells (ie, T cells); and (3) myeloid cells (ie, maturing neutrophils, monocytes, and eosinophils).

†Myelodysplastic/myeloproliferative disorder.

‡Chronic myelomonocytic leukemia.

§Acute myeloid leukemia.

||Myelodysplastic/myeloproliferative disorder and multiple myeloma.

levels in patients with ISM and those with ASM. Patients with ISM showed a lower number of altered (all upregulated) genes (n = 479) than patients with ASM (n = 677 deregulated genes; 674 upregulated and 3 downregulated). More than 50% of the deregulated genes (n = 398 genes) were altered in common in the 2 subgroups of SM, comprising over 80% of the genes deregulated in patients with ISM. It is noteworthy that the IL-2 receptor α chain gene (*IL2RA*; CD25) was significantly upregulated in both groups of patients (fold-change systematically, >13.0).

Functional characterization of the deregulated GEPs common to BMMCs from patients with ISM and those with ASM

Analysis of the biological and functional significance of the deregulated GEPs common to BMMCs carrying the D816V *KIT* mutation (ie, those 398 genes significantly altered in both patients with ISM and patients with ASM) revealed a significant enrichment for genes involved in biological functions mostly related to the immune response (Fig 1). This included genes involved

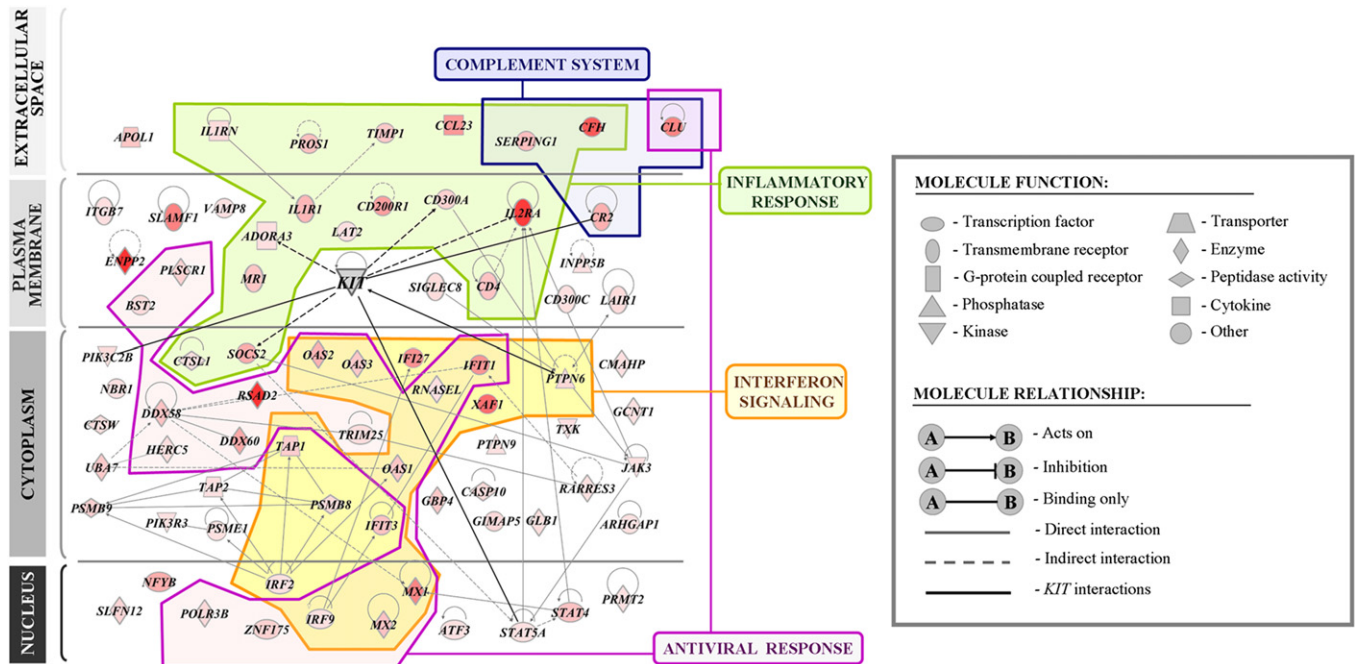


FIG 1. Altered GEPs common to the distinct subtypes of SM (ISM and ASM): network pathway analysis of those genes that have been associated with the immune response, that have been previously described to be regulated by or to interact with *KIT*, or both. Upregulated genes are depicted in red; the color intensity reflects the fold-change levels versus those observed in normal/reactive BMMCs.

in innate and inflammatory immune responses, such as IL-1 receptor type I (*IL1R1*), the *CCL23* chemokine, the *CD4* glycoprotein, and complement-associated (regulatory) molecules, such as clusterin (*CLU*), complement factor H (*CFH*), and plasma protease C1 inhibitor (*SERPING1*; Fig 1 and Table II).²¹ Additionally, expression of genes related to signal transduction associated with cytokine-mediated signaling (Table II), such as Janus kinase/signal transducer and activator of transcription (STAT) pathway genes (eg, *STAT5A* and *STAT4*) and their downstream targets suppressor of cytokine signaling 2 (*SOC2*) and the *PIM1* oncogene, was also upregulated in common in patients with ISM and patients with ASM (Table II). Furthermore, genes associated with type I interferon signaling, together with genes involved in antiviral responses (Fig 1 and Table II) were also altered in BMMCs from both groups of SM, as reflected by upregulation of the *MX1*, *MX2* (interferon-induced GTP-binding proteins Mx1 and Mx2), *OAS1*, *OAS2*, and *OAS3* genes (2'-5'-oligoadenylate synthetases 1, 2 and 3), *IFIT1* and *IFIT3* (interferon-induced protein with tetratricopeptide repeats 1 and 3), and cytosolic DNA-sensing pathway genes, such as the *DDX58* (DEAD [Asp-Glu-Ala-Asp] box polypeptide 58) enzyme, respectively (Table II). Furthermore, marked upregulation of the transcriptional machinery (including initiation, elongation, and termination) and mRNA capping-related genes (eg, the general transcription factor IIa subunit 1 [*GTF2A1*], general transcription factor IIH subunit 1 [*GTF2H1*], general transcription factor IIE subunit 1 [*GTF2E1*], general transcription factor IIH subunit 3 [*GTF2H3*], and excision repair cross-complementing rodent repair deficiency, complementation group 3 [*ERCC3*] transcription factors) in association with transcripts involved in protein-trafficking, such as the N-ethylmaleimide-sensitive factor attachment protein α (*NAPA*) and Golgi-associated PDZ and coiled-coil

motif containing (*GOPC*) genes, was also observed in patients with both disease variants. Similarly, upregulation of genes related to lipid metabolism, including molecules involved in lipid transport, such as the apolipoprotein L family genes *APOL1*, *APOL2*, *APOL4*, and *APOL6* (apolipoprotein L1, L2, L4, and L6) and metabolic enzymes, such as lysosomal acid lipase/cholesterol ester hydrolase [*LIPA*], Table II), was also detected.

Functional profile of genes differentially expressed in BMMCs from patients with ISM and patients with ASM

Functional enrichment analysis of the distinct GEPs associated with the 2 major subtypes of SM revealed only a few specifically deregulated biological functions in patients with ISM. These included upregulated proteins classically associated with signaling pathways involved in MC degranulation, such as linker for activation of T cells (*LAT*) and *LAT*, member 2 (*LAT2*, also known as *NTAL*), together with significant enrichment in genes involved in cell adhesion, including the Siglec-3 (*CD33*) and cadherin 12 (*CDH12*) genes, and the *CD81* tetraspanin was specifically upregulated in patients with ISM (Table III).²¹ Conversely, patients with ASM displayed a more deregulated GEP, with significant upregulation of genes related with apoptosis, including the caspase 1 (*CASP1*), caspase 10 (*CASP10*), XIAP-associated factor 1 (*XAF1*), tumor necrosis factor receptor superfamily member 10B, also known as TRAIL-R2 (*TNFRSF10B*), interleukin 1 beta (*IL1B*), and oncostatin M (*OSM*) genes (Table IV).²¹ Additionally, BMMCs from patients with ASM also displayed significant deregulation of multiple cell cycle-related genes, including transcripts involved in the G₁/S transition, S-phase, and cell-cycle checkpoints, together with genes involved in DNA repair: these included the cyclin

TABLE II. Functional profile of those genes differentially expressed on *KIT* mutated BMMCs from both patients with ISM and those with ASM versus normal/reactive BMMCs (n = 398 genes)

GO code	Functional category	No. of genes	Gene %*	Corrected P value†	Gene ID
0006355	Regulation of transcription, DNA-dependent	36	2.3	.02	<i>TFB1M, ZNF25, NCOA7, ZNF507, ZNF490, IRF2, HLTf, ZNF616, ZNF792, ZNF559, CTCF, GTF2H3, ARRB1, ATF3, ZBTB41, ZNF138, ZNF670, ATF7, VIPAR, ZNF175, GTF2A1, GTF2E1, KIAA1826, IRF9, RNF14, GTF2H1, STAT4, ZNF597, FADS1, NFYB, C1orf103, ERCC3, ZNF701, EYA3, ZFP62, TRIM25</i>
0007165	Signal transduction	27	2.4	.04	<i>ZDHHC13, LGALS3BP, NRP1, SIGLEC8, MYO10, ADORA3, MX1, GRB10, ARRB1, ITPK1, ICK, CD4, PTPN6, AKAP12, SHISA5, NEK6, ARHGAP1, RNF14, IL1R1, INPP4B, SNX17, CCL23, PDE3A, INPP5B, TMEM9B, BST2, PRMT2</i>
0006810	Transport	19	3.2	.01	<i>APOL2, SLC35A1, TXNRD3, UQCRCQ, SLC25A30, TMED8, NDUFS5, APOL6, APOL4, CTNS, APOL1, SLC25A46, FKBP15, FRRS1, NDUFA4, CROT, FADS1, THOC5, NDUFS2</i>
0019221	Cytokine-mediated signaling pathway	17	11.5	.0001	<i>RNASEL, XAF1, OAS3, IFI27, IRF2, STAT5A, MX1, OAS1, MT2A, MX2, JAK3, PTPN6, IRF9, OAS2, STAT4, IFIT3, IFIT1</i>
0006468	Protein phosphorylation	14	3.7	.02	<i>RNASEL, TESK2, GTF2H3, GOLGA5, JAK3, ICK, NEK6, ALPK1, GTF2H1, STAT4, ERCC3, PIM1, TXK, POLR2H</i>
0005975	Carbohydrate metabolic process	13	4.7	.007	<i>NUP37, SLC35A1, IDH1, UEVLD, PHKA1, TPI1, GLB1, AAAS, SLC3A2, MDH2, FBP1, MLEC, GPD1L</i>
0060337	Type I interferon-mediated signaling pathway	13	22.0	<.0001	<i>RNASEL, XAF1, OAS3, IFI27, IRF2, MX1, OAS1, MX2, PTPN6, IRF9, OAS2, IFIT3, IFIT1</i>
0006955	Immune response	12	3.8	.03	<i>CR2, OAS3, IL1RN, IL2RA, OAS1, CD4, ENPP2, OAS2, CTSW, IL1R1, CCL23, MRL</i>
0045087	Innate immune response	12	4.4	.01	<i>CR2, APOL1, HERC5, CLU, DDX58, JAK3, SERPING1, IL1R1, BST2, UBA7, CASP10, TRIM25</i>
0016032	Viral reproduction	11	3.5	.05	<i>NUP37, PSME1, GTF2H3, RPL39, AAAS, CD4, GTF2A1, GTF2E1, GTF2H1, ERCC3, POLR2H</i>
0030168	Platelet activation	11	4.8	.01	<i>PROS1, TIMP1, ARRB1, CLU, PTPN6, ITGA2B, SERPING1, PIK3R3, PDE3A, SLC8A3, PLSCR1</i>
0009615	Response to virus	10	7.2	.002	<i>MX1, HERC5, MX2, CLU, ZNF175, POLR3B, IRF9, IFIT1, DDX60, TRIM25</i>
0055114	Oxidation-reduction process	10	4.7	.02	<i>IDH1, ACOX1, KDM5D, MTHFD1, FADS1, HTATIP2, TBXAS1, OGDH, NDUFS2, GPD1L</i>
0006917	Induction of apoptosis	8	4.4	.04	<i>MX1, ZMAT3, SHISA5, CIDEB, HTATIP2, ERCC3, PRMT2, CASP10</i>
0006368	Transcription elongation from RNA polymerase II promoter	7	11.3	.002	<i>GTF2H3, GTF2A1, GTF2E1, GTF2H1, ERCC3, ELP3, POLR2H</i>
0006464	Protein modification process	7	4.8	.05	<i>SLC35A1, UEVLD, ST8SIA4, HERC5, PADI2, UBA7, HERC6</i>
0016042	Lipid catabolic process	7	7.3	.01	<i>LIPA, RARRES3, PAFAH2, PLD3, ENPP2, ABHD4, PLCB2</i>
0060333	IFN- γ -mediated signaling pathway	7	12.1	.002	<i>OAS3, IRF2, OAS1, MT2A, PTPN6, IRF9, OAS2</i>

(Continued)

TABLE II. (Continued)

GO code	Functional category	No. of genes	Gene %*	Corrected P value†	Gene ID
0006367	Transcription initiation from RNA polymerase II promoter	6	9.2	.01	<i>GTF2H, GTF2A1, GTF2E1, GTF2H1, ERCC3, POLR2H</i>
0051607	Defense response to virus	6	13.3	.003	<i>RNASEL, OAS3, OAS1, RSAD2, BST2, PLSCR1</i>
0006091	Generation of precursor metabolites and energy	5	8.9	.02	<i>ACOX1, PHKA1, CROT, COX17, OGDH</i>
0007059	Chromosome segregation	5	8.6	.03	<i>NUP37, MIS12, CTCF, NEK6, NSL1</i>
0009611	Response to wounding	5	7.8	.04	<i>NRP1, ADORA3, CLU, PTPN6, DST</i>
0045071	Negative regulation of viral genome replication	5	31.3	.0004	<i>RNASEL, OAS3, OAS1, IFIT1, PLSCR1</i>
0006094	Gluconeogenesis	4	9.3	.04	<i>TP11, ATF3, MDH2, FBP1</i>
0006099	Tricarboxylic acid cycle	4	17.4	.01	<i>IDH1, MDH2, OGDH, SUCLG2</i>
0006120	Mitochondrial electron transport, NADH to ubiquinone	4	10.3	.04	<i>NDUFS5, NDUFAF, NDUFA4, NDUFS2</i>
0006283	Transcription-coupled nucleotide-excision repair	4	9.3	.04	<i>GTF2H3, GTF2H1, ERCC3, POLR2H</i>
0006360	Transcription from RNA polymerase I promoter	4	17.4	.01	<i>GTF2H3, GTF2H1, ERCC3, POLR2H</i>
0006361	Transcription initiation from RNA polymerase I promoter	4	17.4	.01	<i>GTF2H, GTF2H1, ERCC3, POLR2H</i>
0006362	Transcription elongation from RNA polymerase I promoter	4	22.2	.007	<i>GTF2H3, GTF2H1, ERCC3, POLR2H</i>
0006363	Termination of RNA polymerase I transcription	4	20.0	.009	<i>GTF2H3, GTF2H1, ERCC3, POLR2H</i>
0006370	mRNA capping	4	14.3	.02	<i>GTF2H3, GTF2H1, ERCC3, POLR2H</i>
0006888	ER to Golgi vesicle-mediated transport	4	9.1	.04	<i>GOPC, TRAPPC3, BET1, ERGIC1</i>
0007259	JAK-STAT cascade	4	14.8	.01	<i>STAMBP, STAT5A, STAT4, SOCS2</i>
0032088	Negative regulation of NF- κ B transcription factor activity	4	9.8	.04	<i>ARRB1, TRIM21, DAP, PRMT2</i>
0032480	Negative regulation of type I interferon production	4	13.3	.02	<i>HERC5, DDX58, UBA7, TRIM25</i>
0042157	Lipoprotein metabolic process	4	10.8	.03	<i>APOL2, APOL6, APOL4, APOL1</i>
0050434	Positive regulation of viral transcription	4	9.1	.04	<i>GTF2H3, GTF2H1, ERCC3, POLR2H</i>
0000718	Nucleotide-excision repair, DNA damage removal	3	15.0	.04	<i>GTF2H3, GTF2H1, ERCC3</i>
0006103	2-oxoglutarate metabolic process	3	15.8	.04	<i>IDH1, STAT5A, ADHFE1</i>
0006164	Purine nucleotide biosynthetic process	3	16.7	.04	<i>OAS1, MTHFD1, OAS2</i>
0042104	Positive regulation of activated T cell proliferation	3	17.6	.03	<i>STAT5A, IL2RA, JAK3</i>
0043029	T cell homeostasis	3	16.7	.04	<i>STAT5A, GIMAP5, JAK3</i>
0019853	L-ascorbic acid biosynthetic process	2	100.0	.01	<i>AKR1A1, GSTO1</i>
0021860	Pyramidal neuron development	2	40.0	.04	<i>UQCRCQ, OGDH</i>
0032020	ISG15-protein conjugation	2	33.3	.04	<i>HERC5, UBA7</i>
0035457	Cellular response to IFN- α	2	50.0	.03	<i>OAS1, IFIT3</i>
0045588	Positive regulation of $\gamma\delta$ T cell differentiation	2	33.3	.04	<i>STAT5A, GIMAP5</i>
0050688	Regulation of defense response to virus	2	33.3	.04	<i>HERC5, IFIT1</i>
0060700	Regulation of ribonuclease activity	2	100.0	.01	<i>OAS3, OAS1</i>

GO, Gene Ontology.

*The percentage of genes within a functional category is presented as the ratio between the number of genes differentially expressed in the GEP common to all diagnostic subtypes of SM assigned to a GO term and the total number of genes included in the array, which are annotated to the same GO term.

†P value corrected for multiple hypothesis testing by using the false discovery rate method of Benjamini and Hochberg, as defined in Carmona-Saez et al.²¹

A1 (*CCNA1*), cyclin B1 (*CCNB1*), CDC28 protein kinase regulatory subunit 2 (*CKS2*), and cyclin-dependent kinase 4 (*CDK4*) cell cycle-associated genes and the proliferating cell nuclear antigen (*PCNA*) and polymerase (DNA-directed), delta 3, accessory subunit (*POLD3*) DNA repair genes, among others (Table IV).

From a practical point of view, a set of only 4 genes (*CCL23*, *IL13*, *IL1B*, and *CR2*) was identified by means of

supervised class prediction (PAM) analysis, the combination of which allowed for the classification of patients with ISM versus patients with ASM (Fig 2) with an overall accuracy of 96%, with only 1 patient with ISMs+ (SSM variant) with myeloid plus lymphoid multilineage *KIT* D816V mutation (case 19, Table I) being consistently misclassified (see the Results section and Table E2 in this article's Online Repository at www.jacionline.org).

TABLE III. Functional profile of those genes differentially expressed by BMMCs from patients with ISM versus both normal/reactive BMMCs and BMMCs from patients with ASM (n = 479)

GO code	Functional category	No. of genes	Gene %*	Corrected P value†	Gene ID
0007155	Cell adhesion	19	3.7	.02	<i>LGALS3BP, CD33, NRP1, SIGLEC8, COL12A1, CD300A, MPZL2, CLSTN1, PERP, CD4, ITGA2B, FERMT3, MPZL3, NTM, CDH12, FREM1, ITGB7, DST, INPPL1</i>
0055085	Transmembrane transport	19	3.1	.05	<i>SFXN1, SLC30A6, SLC39A11, NUP37, SLC35A1, ABCC5, TPCN1, SCN7A, AAAS, SLC3A2, SLC45A3, ADCY7, KCNAB2, DST, ATP6V0B, CLCN7, SLC46A3, SLC8A3, SCN2A</i>
0007596	Blood coagulation	16	3.6	.04	<i>PROS1, IRF2, TIMP1, ARRB1, ITPK1, CLU, SLC3A2, PTPN6, STX4, ITGA2B, SERPING1, PIK3R3, PDE3A, LAT, HIST1H3A, SLC8A3</i>
0006886	Intracellular protein transport	9	4.8	.05	<i>STON2, MLPH, AP1G2, ARFIP1, STX4, VIPAR, NAPA, SNX17, KDELR1</i>
0035335	Peptidyl-tyrosine dephosphorylation	5	8.6	.04	<i>PTPN5, PTPN6, PTPN9, DUSP3, EYA3</i>
0006605	Protein targeting	4	12.9	.03	<i>STXBP4, MLPH, AKAP12, PML</i>
0006749	Glutathione metabolic process	4	12.9	.03	<i>IDH1, CTNS, GSTM2, GSTM3</i>
0006892	Post-Golgi vesicle-mediated transport	4	10.3	.05	<i>VAMP8, ARRB1, STX4, NAPA</i>
0050868	Negative regulation of T cell activation	3	33.3	.02	<i>GIMAP5, JAK3, DUSP3</i>
0006290	Pyrimidine dimer repair	2	66.7	.02	<i>POLH, DDB2</i>
0021860	Pyramidal neuron development	2	40.0	.05	<i>UQCRCQ, OGDH</i>
0032288	Myelin assembly	2	66.7	.02	<i>PMP22, ILK</i>
0032957	Inositol trisphosphate metabolic process	2	66.7	.02	<i>ITPK1, INPPL1</i>
0042178	Xenobiotic catabolic process	2	50.0	.03	<i>GSTM2, GSTM4</i>
0043303	MC degranulation	2	50.0	.03	<i>LAT2, LAT</i>
0043570	Maintenance of DNA repeat elements	2	40.0	.05	<i>AXIN2, MSH2</i>
0070602	Regulation of centromeric sister chromatid cohesion	2	66.7	.02	<i>AXIN2, CTCF</i>

GO, Gene Ontology.

*The percentage of genes within a functional category is presented as the ratio between the number of genes differentially expressed in the GEP common to all diagnostic subtypes of SM assigned to a GO term and the total number of genes included in the array, which are annotated to the same GO term.

†P value corrected for multiple hypothesis testing using the false discovery rate method of Benjamini and Hochberg, as defined in Carmona-Saez et al.²¹

Validation of GEP microarray data by using multiparameter flow cytometric immunophenotyping

Validation studies performed at the protein level in the same group of patients used for GEP microarray analyses showed the existence of a significant correlation between gene expression data (unlogged arbitrary intensity units) and protein expression (ie, mean expression intensity expressed as mean fluorescence intensity [in arbitrary units]) for most of the deregulated genes tested: *IL2RA* ($r^2 = 0.9$, $P < .0001$, $n = 12$), *CD4* ($r^2 = 0.7$, $P = .015$, $n = 7$), and *IL3RA* ($r^2 = 0.5$, $P = .0002$, $n = 22$) but not for *HLA-DRB5*, which did not exhibit a significant correlation with HLA-DR protein levels. Interestingly, despite the fact that *CD2* expression was not significantly deregulated at the mRNA level in BMMCs from patients with ISM or patients with ASM, a significant correlation was observed for this marker between gene and protein expression levels ($r^2 = 0.5$, $P = .007$, $n = 21$).

Further validation of the GEPs in a second independent and larger cohort of patients ($n = 176$) showed overall similar profiles for gene and protein expression for *CD21*, *CD25*, *CD33*, *CD52*, and *LAIR1* (*CD305*; Fig 3). Interestingly, despite the fact that the *CD4* gene was upregulated in the common GEP, significantly increased protein expression levels were mostly detected among patients with ISM lacking skin lesions ($P \leq .0001$); similarly, no differences were found for *CD2* expression at the mRNA level between patients with ISM and those with ASM, although this marker was significantly upregulated at the protein level, particularly among patients with ISMs— (Fig 3). Interestingly,

upregulation of the *HLA-DRB5*, *FCGR2B*, and *IL3RA* genes was exclusively observed in patients with ASM, whereas all subtypes of SM analyzed displayed overall increased levels of these proteins in this validation set ($P < .0001$). Nevertheless, although *IL3RA* (*CD123*) protein expression was found at variable frequencies in patients with ISMs— (14/21 [67%]) and patients with ISMs+ (22/48 [46%]) versus patients with ASM (7/7 [100%]); positivity for HLA-DR and *CD32* was similarly distributed among all variants of the disease.

Cell-cycle analysis revealed an overall slightly increased proliferation index (PI; percentage of S plus G₂/M cells) of BMMCs among all SM subtypes analyzed ($P < .02$ vs normal/reactive BMMCs), although cases with high proliferation (PI >5%) were only found among patients with ASM (2/5 [40%] cases, Fig 3).

DISCUSSION

SM is a clinical, biological, and prognostically heterogeneous group of disorders characterized by an abnormal expansion and accumulation of clonal MCs in different tissues, including BM.² Because the majority (>90%) of patients with SM carry the same genetic lesion (D816V *KIT* mutation),⁹ the *KIT* mutation itself does not explain the heterogeneous clinical behavior of the disease, and the molecular mechanisms and pathways underlying the different subtypes of SM remain largely elusive. To address this issue, we analyzed, for the first time, the GEPs of highly purified MCs isolated from the BM of patients with SM with distinct

TABLE IV. Functional profile of those genes differentially expressed by BMMCs from patients with ASM versus both normal/reactive BMMCs and BMMCs from patients with ISM (n = 677)

GO code	Functional category	No. of genes	Gene %*	Corrected P value†	Gene ID
0006915	Apoptosis	28	4.8	.01	<i>FASTKD1, APH1A, XAF1, PSMB3, NMT1, PSME1, IL1B, NCSTN, IL2RA, GADD45A, ZMAT3, NLRP2, TPX2, PTPN6, TCTN3, SHISA5, NEK6, CIDEA, CASP1, ZC3H12A, BFAR, RNF34, GADD45B, OSM, TNFRSF10B, CASP10, PLSCR1, SCN2A</i>
0000278	Mitotic cell cycle	19	6.2	.006	<i>PCNA, POLD3, NUP37, CCNA1, PSMB3, MIS12, SKP2, PSME1, CDK4, DCTN2, DYNC1I2, CENPJ, TYMS, RFC2, MCM7, NSL1, CDC23, CCNB1, PRIM1</i>
0008283	Cell proliferation	17	5.7	.02	<i>PCNA, SKP2, IRF2, CKS2, IL2RA, DCTN2, TPX2, DAB2, ENTPD5, STAT4, MCM7, WDR12, UBE2V2, UBE2L3, PIM1, BST2, OSM</i>
0006366	Transcription from RNA polymerase II promoter	15	5.2	.04	<i>TRIP11, EGR2, GTF2H3, ARRB1, CPSF3, GTF2A1, GTF2E1, IRF9, GTF2H1, TRIP4, CSTF3, ERCC3, TADA2A, POLR2H, TSC22D1</i>
0006457	Protein folding	14	7.9	.006	<i>FKBP14, PPIF, CCT3, EDEM2, TOR1A, DNAJB9, DNAJC24, AARS, FKBP15, AHS1, UXT, MPDU1, DNAJC16, MLEC</i>
0000082	G1/S transition of mitotic cell cycle	10	7.0	.03	<i>PCNA, CCNA1, PSMB3, SKP2, PSME1, CDK4, TYMS, MCM7, CCNB1, PRIM1</i>
0016567	Protein ubiquitination	10	6.8	.04	<i>MARCH2, UBE2L6, ARRB1, TRIM21, RNF111, DCAF7, RNF14, CDC23, UBE2V2, UBE2L3</i>
0043123	Positive regulation of IκB kinase/NF-κB cascade	10	7.5	.02	<i>ZDHHC13, IL1B, SHISA5, NEK6, TLR3, CASP1, TMEM9B, BST2, TNFRSF10B, CASP10</i>
0000084	S phase of mitotic cell cycle	9	8.1	.02	<i>PCNA, POLD3, PSMB3, SKP2, PSME1, CDK4, RFC2, MCM7, PRIM1</i>
0022904	Respiratory electron transport chain	8	8.8	.03	<i>UQCRCQ, NDUFA2, NDUFS5, ATP5E, COX6B1, NDUFA4, ATP5J2, NDUFS2</i>
0071260	Cellular response to mechanical stimulus	8	16.3	.003	<i>IL13, IL1B, GADD45A, CASP8AP2, TLR3, CASP1, MAP2K4, TNFRSF10B</i>
0000079	Regulation of cyclin-dependent protein kinase activity	7	12.7	.01	<i>BLM, CCNA1, CKS2, GADD45A, HERC5, GTF2H1, CCNB1</i>
0006139	Nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	7	10.6	.02	<i>OAS3, DGUOK, TPMT, OAS2, TYMS, PRPSAP1, AK2</i>
0006289	Nucleotide-excision repair	7	10.6	.02	<i>PCNA, POLD3, GTF2H3, GTF2H1, RFC2, ERCC3, POLR2H</i>
0006633	Fatty acid biosynthetic process	7	13.0	.01	<i>PRKAB1, ACSM3, SC4MOL, LIPC, TBXAS1, AASDHPPT, CBR4</i>
0006919	Activation of caspase activity	7	10.0	.03	<i>IFI27, NLRP3, AIFM1, CASP8AP2, DAP, CASP1, TNFRSF10B</i>
0006271	DNA strand elongation involved in DNA replication	5	16.1	.02	<i>PCNA, POLD3, RFC2, MCM7, PRIM1</i>
0015992	Proton transport	5	11.9	.05	<i>ATP6AP1, ATP5E, ATP6V1E1, ATP6V0B, ATP5J2</i>
0000722	Telomere maintenance via recombination	4	17.4	.04	<i>PCNA, POLD3, RFC2, PRIM1</i>
0009083	Branched chain family amino acid catabolic process	4	23.5	.02	<i>BCKDHB, HIBADH, MCCC2, DBT</i>
0009311	Oligosaccharide metabolic process	4	33.3	.01	<i>MGAT2, ST8SIA4, ST8SIA6, NAGA</i>
0032201	Telomere maintenance via semi-conservative replication	4	18.2	.04	<i>PCNA, POLD3, RFC2, PRIM1</i>
0006154	Adenosine catabolic process	2	66.7	.04	<i>ADAL, CECR1</i>
0009597	Detection of virus	2	66.7	.04	<i>DDX58, TLR3</i>
0034638	Phosphatidylcholine catabolic process	2	100.0	.02	<i>LIPC, ENPP2</i>
0042506	Tyrosine phosphorylation of Stat5 protein	2	100.0	.02	<i>JAK3, OSM</i>
0046103	Inosine biosynthetic process	2	66.7	.04	<i>ADAL, CECR1</i>

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†P value corrected for multiple hypothesis testing using the false discovery rate method of Benjamini and Hochberg, as defined in Carmona-Saez et al.²¹

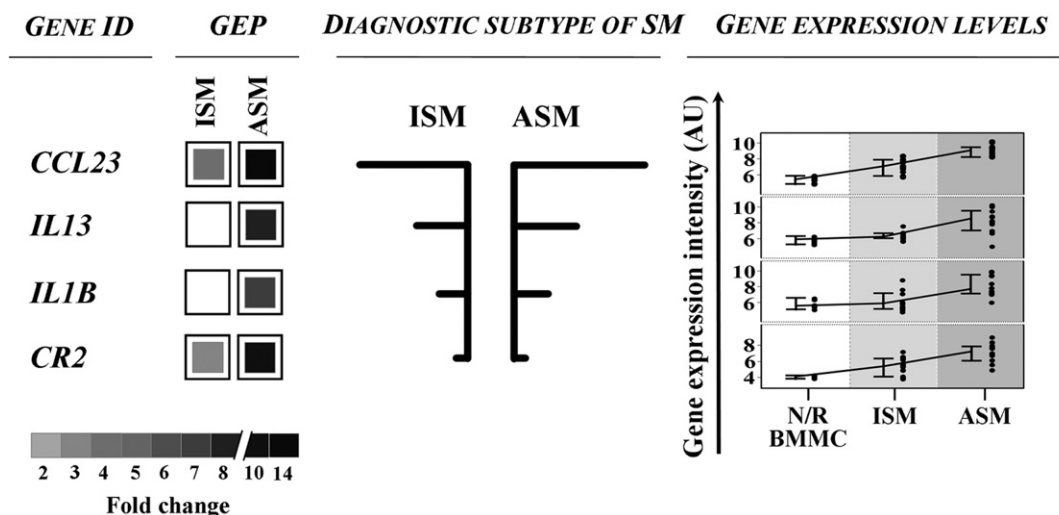


FIG 2. Discrimination between patients with ISM and those with ASM based on a restricted number of genes differentially expressed by BMMCs. Gene expression levels (GEPs) of those genes that contributed to the discrimination between ISM and ASM are shown by color codes in the *left column*. Centroid plots are shown in the *middle column*, whereas gene expression charts on the right display medians $\pm$ 95% CIs. AU, Log-transformed fluorescence arbitrary units; N/R BMMC, normal/reactive BMMCs.

variants of the disease carrying the D816V *KIT* mutation. Overall, our results show that the distinct subtypes of SM share a common GEP that is distinct from that of normal/reactive BMMCs; in turn, distinct expression and functional profiles were also detected in patients with ISM versus patients with ASM.

Stem cell factor signaling through its receptor, c-Kit, plays a key role in the biology of MCs, leading to the engagement of multiple downstream signaling pathways involved in MC growth, differentiation, survival, chemotaxis, and cytokine production.^{25,26} Interestingly, the activating D816V *KIT* mutation, which is common to all patients with SM studied here, translated into a common GEP characterized by generalized upregulation of genes involved in cytokine-mediated signal transduction, including target genes of the Janus kinase/STAT pathway, a c-Kit downstream signaling pathway described to be activated in D816V⁺ MCs.²⁷ Within this common GEP, a significant enrichment was found for type I interferon-dependent immune response genes in the absence of interferon therapy. Because most of these interferon-induced genes (eg, *OAS1*, *OAS2*, *MX1*, *MX2*, or *IRF9*) have been found to be also up-regulated on MC infection by viruses,^{28,29} our results could suggest that c-Kit downstream signaling could play an important role in MC responses to viral infection. In line with this hypothesis, previous studies have reported that infection of endothelial cells by the Kaposi sarcoma herpesvirus induces upregulation of c-Kit and that the inhibition of either c-Kit activation or its downstream effectors reverses the characteristic Kaposi sarcoma herpesvirus-induced morphologic transformation of infected cells.^{30,31}

Furthermore, an overall enrichment in genes involved in the innate and inflammatory immune response was also detected in both patients with ISM and those with ASM. These included genes coding for complement regulatory proteins, such as *CFH*, *SERP-ING1*, and *CLU*, with the latter previously reported to be up-regulated also in BM mononuclear cells from patients with SM.¹² These findings, together with the increased expression of membrane-bound complement inhibitory proteins, such as CD35 (complement receptor 1) and CD59 (membrane inhibitor of reactive lysis),^{8,32} could reflect the involvement of c-Kit signaling in patients with SM in the enhancement of self-defense mechanisms

against complement-dependent cytotoxicity, which is similar to what has been described for other types of tumor cells.³³ Functional enrichment analysis of the GEP common to both the indolent and aggressive forms of the disease also revealed significant upregulation of transcripts involved in lipid metabolism, probably translating to an increased phospholipid metabolism and production of arachidonic acid metabolites (eg, prostaglandin D₂ and leukotriene C₄),^{34,35} and in transcription and protein processing (probably reflecting an increased rate of *de novo* production of MC-associated proteins as a result of the presence of an activating *KIT* mutation),^{25,36} which could be partially responsible for the MC mediator-related symptoms typically displayed by patients with SM.^{4,6}

Interestingly, in addition to the common GEP, BMMCs from patients with ISM and those with ASM also showed different GEPs. Because the D816V *KIT* mutation was a common feature to all patients with SM analyzed, the heterogeneous clinicobiological features observed in patients with ISM versus those with ASM could result from, for example, coexistence of additional distinct genetic lesions among the later cases. In this regard our results point out the existence in BMMCs from patients with ASM but not those from patients with ISM of a significant deregulation of 2 distinct but interconnected cell functions. The first is impaired apoptosis (reflected by upregulation of antiapoptotic genes, such as the bifunctional apoptosis regulator [*BFAR*]), and the second is cell-cycle deregulation associated with increased expression of genes involved in cell-cycle checkpoints, such as cyclin B1 (*CCNB1*), which is often described to be over-expressed in various human tumors³⁷⁻³⁹ in association with an increased cell proliferation.⁴⁰ In line with these observations, although a slight increase in BMMC proliferation was observed for all variants of SM analyzed, only BMMCs from patients with ASM exhibited a high PI. Altogether, these findings suggest that different mechanisms leading to either increased proliferation, inhibition of apoptosis, or both might be involved in the pathogenesis of ASM and could contribute to explaining the heterogeneous clinicobiological behavior^{1,2} and variable response to treatment⁴¹ of these patients. In line with this hypothesis, *NRAS*

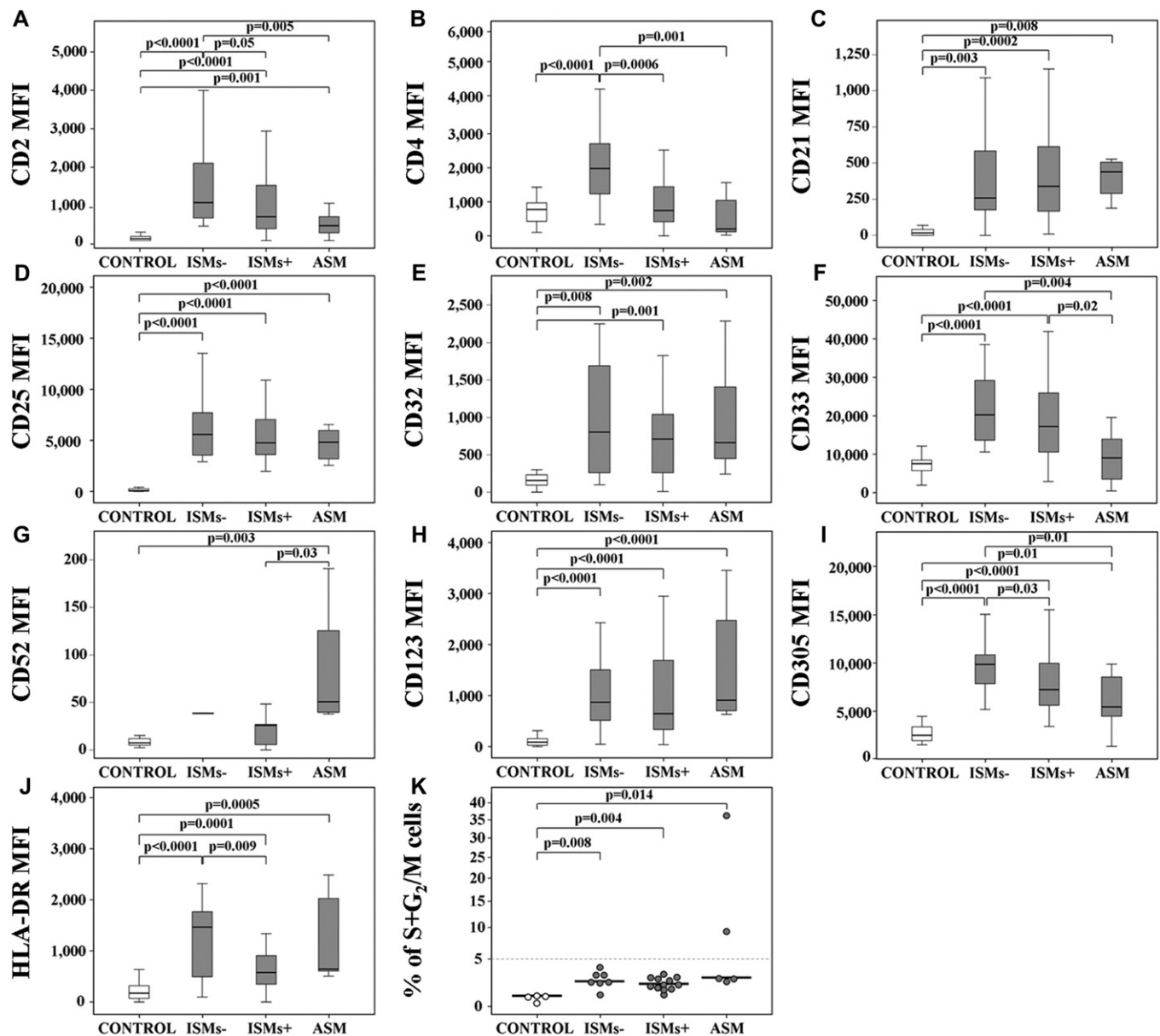


FIG 3. Immunophenotypic characteristics (A-J; mean fluorescence intensity [MFI] values per MC) and PI values (percentage of S plus G₂/M cells) of BMMCs (K) from patients with SM compared with normal/reactive BMMCs (CONTROL).

and *TET2* mutations have been previously reported in relatively small fractions of patients with ASM.^{42,43} Further studies in larger cohorts of patients are required to confirm the existence of distinct GEPs within BMMCs from patients with ASM and their potential association with both specific genetic lesions other than the D816V *KIT* mutation and distinct clinical behavior of the disease.

In contrast to patients with ASM, patients with ISM displayed few specifically deregulated genes (>80% of the genes deregulated in patients with ISM were also altered in patients with ASM). This could be due to the fact that those patients with ISM here analyzed displayed a heterogeneous BMMC burden, together with variable clinical (ISM⁻ vs typical ISM⁺ vs SSM) and molecular (*KIT* mutation restricted to the MC compartment vs multilineage involvement) features, some of which have been suggested to have a significant effect in the protein

expression profile of BMMCs of patients with ISM.⁴⁴ Despite all of the above, patients with ISM displayed significant upregulation of adhesion-related molecules, such as *CD33*, *CDH12*, or the sialic acid binding Ig-like lectin 8 (*SIGLEC8*), when compared with both control subjects and patients with ASM. Because ISM variants typically involve the BM together or not with the skin, whereas ASM frequently infiltrates other organs and tissues (eg, spleen, liver, lymph nodes, and/or peripheral blood),^{1,2} such a distinct pattern of expression of adhesion-related genes could potentially contribute to explaining the different patterns of organ/tissue involvement in both groups of patients with SM.

Interestingly, despite the clinical, biological, and molecular heterogeneity observed among patients with ISM, a GEP signature based on only 4 genes allowed for the distinction between patients with ISM and those with ASM; except for 1 patient with

ISMs+ (SSM) that recently evolved to mast cell leukemia, this included the discrimination between patients with ASM and patients with ISMs+ with multilineage BM involvement (a subgroup of patients with ISM that has been previously described to exhibit immature immunophenotypic features for BMMCs similar to those of patients with ASM).⁴⁴ Because progression of cases of ISMs+ with multilineage BM involvement by the *KIT* D816V mutation to ASM is not a rare event,⁴⁵ our results suggest that this gene signature could also potentially serve in the future as prior knowledge for the discovery of biomarker candidates (eg, serum CCL23, IL-1 β , or IL-13 levels) for monitoring patients with ISM with multilineage BM involvement by the *KIT* mutation and higher risk of disease progression.

Despite all of the above, a major concern remains regarding the functional effect of mRNA levels on protein expression. Here we found a significant positive correlation between mRNA and protein levels for the majority of the genes tested, except for *HLA-DRB5* (in the 2 validation groups) and *FCGR2B* (in the second validation set). These results are most likely due to the fact that the mAbs used to validate the expression of these 2 genes also recognize other paralogs/isoforms of these proteins and further suggest that such distinct paralogs/isoforms for HLA-DR and CD32 might be differently upregulated in individual patients with SM.

In summary, here we show, for the first time, that the D816V activating *KIT* mutation confers a common GEP to BMMCs from distinct subvariants of SM, characterized by upregulation of genes involved in innate and inflammatory immune responses, including interferon-induced genes involved in antiviral responses, as well as genes involved in both lipid and protein metabolism. In addition, distinct and unique GEPs were also observed in indolent versus aggressive variants of SM, which reflect increased MC adhesion in patients with ISM and altered apoptosis, cell-cycle regulation, or both in patients with ASM and highlight the potential nature of relevant secondary genetic lesions, microenvironmental alterations, or both specific for these subgroups of SM.

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Key messages

- The D816V *KIT* mutation in BMMCs from patients with SM is associated with upregulation of genes involved in innate and inflammatory immune responses.
- Different subtypes of SM exhibit distinct GEPs, which reflect deregulation of apoptosis and the cell cycle in patients with ASM and altered adhesion properties in patients with ISM.

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METHODS

Serum tryptase levels

Serum baseline tryptase levels were determined by using the Phadia ImmunoCAP Tryptase System (Phadia, Uppsala, Sweden), according to the manufacturer's instructions.

Isolation of MCs and other subpopulations of BM nucleated cells, DNA extraction, and detection of the D816V *KIT* mutation

Isolation of MCs and other subpopulations of nucleated BM cells was performed within the first 24 hours after sample collection by using a 4-way fluorescence-activated cell sorter (FACSARIA; Becton Dickinson Biosciences, San Jose, Calif) equipped with the FACSDiVa software (Becton Dickinson Biosciences), as previously described. Briefly, before sorting, cells were stained with a 5-color combination of mAbs (ie, CD3/CD45/CD117/CD34/CD14 conjugated with Pacific Blue/fluorescein isothiocyanate/phycoerythrin-cyanin 7/allophycocyanin/allophycocyanin-H7) by using well-established stain-and-then-lyse-and-wash procedures, which have been described elsewhere.^{E1} Isolation of BMMCs, eosinophils, monocytes, neutrophils, CD34⁺ hematopoietic precursor cells, and lymphocytes (purity $\geq 97\%$) was performed according to well-established phenotypic criteria previously described in detail.^{E1}

Genomic DNA was extracted with the GenElute Mammalian Genomic DNA Miniprep (Sigma-Aldrich, St Louis, Mo), NucleoSpin Tissue XS (Macherey-Nagel, Düren, Germany), or the REXtract-N-AmpTM blood PCR kit (Sigma-Aldrich), according to the manufacturer's instructions. Positivity for the D816V *KIT* mutation was determined on BMMCs, eosinophils, monocytes, neutrophils, CD34⁺ hematopoietic precursor cells, and lymphocytes by using a PNA-mediated PCR clamping technique (LightCycler thermocycler; software version 3.5; Roche Diagnostics GmbH, Mannheim, Germany) and the DNA Master Hybridization probes Kit (Roche Diagnostics), followed by sequencing of the PCR products in both directions by using the 5'-CAG CCA GAA ATA TCC TCC TTA CT-3' and 5'-TTG CAG GAC TGT CAA GCA GAG-3' primers (Isogen Life Sciences, Maarsse, The Netherlands), as described in detail elsewhere.^{E1,E2}

RNA extraction, microarray hybridization, and data analysis

Analysis of the GEPs of BMMCs was performed on RNA extracted from purified BMMCs from 33 subjects, including 7 control subjects (5 healthy donors and 2 subjects carrying a B-cell lymphoproliferative disorder who exhibited reactive MC hyperplasia [cases 6 and 7]) and 26 patients with SM (Table 1). For each sample, total RNA was extracted from highly pure ($\geq 97\%$ purity) BMMCs by using either the TRIZOL Reagent (Invitrogen, Carlsbad, Calif) or the NucleoSpin RNA XS kit (Macherey-Nagel), according to the manufacturer's instructions. Purity and integrity of the RNA were assessed for each sample by using an Agilent 2100 Bioanalyzer with either the RNA 6000 Nano-chips or Picochips (Agilent Technologies, Santa Clara, Calif). GEPs were determined by using the Affymetrix GeneChip Human Gene 1.0 ST Array (Affymetrix) by using the hybridization, washing, and laser scanning procedures recommended by the manufacturer.

For data analysis, GEP raw data (Affymetrix.CEL files) were normalized by using the RMA algorithm, which included background correction, quantile normalization, $\log_2$ transformation, and probe set summarization.^{E3} Four sets of filters were used to decrease the risk of false-positive results. Briefly, genes that did not show expression levels of 20 or greater (arbitrary unlogged units) in 10% or more of the samples were removed from further analyses. Two additional filters were used to exclude those genes that could be altered because of changes in the BM microenvironment (eg, altered expression in reactive vs normal BMMCs): (1) genes that displayed heterogeneous expression in the control group defined by a coefficient of variation for unlogged values of greater than 40% and (2) genes that showed higher differences between median values in reactive versus normal MCs than in reactive MCs versus MCs from patients with SM. Additionally, a fourth filter, which was devoted to eliminating the variability because of slight differences in the overall quality of the array hybridization, as assessed by the area under the receiver operating

characteristic (ROC) curve discriminating between positive and negative control probe sets ("Pos-vs-Neg-AUC", Affymetrix quality control), was used based on a combination of a K-means clustering algorithm and a linear regression model. Based in this later filter, those genes that exhibited an expression pattern associated with the quality of the hybridization (clusters of genes in which $>25\%$ displayed a significant correlation [$r^2 \geq 0.6$] with the Affymetrix controls) were excluded from further analysis.

For the identification of the best combination of genes that would allow for the discrimination between patients with ISM and those with ASM, a nearest shrunken centroid algorithm (PAM software version 2.1) was used to build a classifier based on those genes significantly deregulated in patients with ISM, patients with ASM, or both ($n = 758$).^{E4} Validation of the classifier was performed, applying a leave-one-out cross-validation method, in which an individual sample from the original set of patients was used for validation of data, whereas the remaining samples were used as a training group.^{E5} This procedure was repeated so that each patient was used once for the validation data to estimate the performance of the out-of-sample validation. The overall performance of the classifier model was assessed by averaging the error under validation over all the cross-validation steps.^{E5} Those genes found to be informative were further used to build a score-based class prediction algorithm. For this purpose, a corrected fold change was calculated for each patient and gene as the ratio between the fold change (given by the ratio between the target gene unlogged expression value for each patient with the mean unlogged expression value of the control samples) of the target gene and that of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) housekeeping gene. The performance of each gene for the discrimination between patients with ISM and patients with ASM was assessed by using ROC curves, and then a score of 1 was assigned to each of the informative genes ($n = 4$). The sum of the individual score for each of the genes included in the model ($n = 4$) was then calculated for each of the patients, and the most discriminant cutoff for the identification of patients with ISM versus patients with ASM was determined by using ROC curve analysis (see Table E2).

Cell-cycle analyses

Assessment of the cell-cycle distribution of BMMCs (percentage of G₀/G₁ and S plus G₂/M phases) was performed by using a previously described 3-color direct immunofluorescence stain and then lyse and wash technique,^{E6} in which nuclear DNA of single cells was stained with DRAQ5 (Vitro SA, Madrid, Spain) and BMMCs were identified by their unique pattern of expression of CD45 and CD117 (see Table E1). The PI (percentage of S plus G₂/M cells) was assessed by 2 different observers for those cases in which more than 100 BMMCs could be analyzed ($n = 29$) by using the INFINICYT software (Cytognos SL, Salamanca, Spain) and a well-established strategy.^{E6}

Statistical methods

For all continuous variables, the medians, means, and SDs, as well as the ranges and both the 25th and 75th and the 10th and 90th percentiles, were calculated. For categorical variables, frequencies were used. Statistical significance ($P \leq .05$ and false discovery rate correction for multiple comparisons of $<10\%$) was determined by using the nonparametric Kruskal-Wallis and Mann-Whitney *U* tests; a linear regression model was used to explore the degree of correlation between different variables. For all statistical analyses, the SPSS 15.0 (SPSS, Chicago, Ill) and MATLAB R2010b (Mathworks, Natick, Mass) software programs were used.

RESULTS

Class prediction algorithm for the classification of patients with ISM versus those with ASM based on GEPs

Supervised class prediction (PAM) analysis based on those 758 transcripts significantly deregulated in patients with SM identified a combination of 4 genes (*CCL23*, *IL13*, *IL1B*, and *CR2*), which allowed for the classification of patients with ISM versus patients with ASM (Fig 2), with an overall accuracy of 96% (25/26

patients correctly classified). To transform this later classification algorithm into a more suitable model for clinical use, a score-based prediction algorithm was built by using these later 4 informative genes (see [Table E2](#)), which identified patients with ASM as those cases with a higher score (≥ 3), with a high sensitivity (92%), specificity (100%), and overall accuracy (96%), with only 1 patient with ASM (case 27, [Table I](#)) being misclassified.

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TABLE E1. Antibody reagents used for the immunophenotypic and cell-cycle analyses of BMMCs

Purpose	Gene	FC ISM	FC ASM	Antibody conjugate	Clone	Source
BMMC identification	NA	NA	NA	CD34-PerCP Cy5.5	8G12	BD Biosciences*
	NA	NA	NA	CD45-Pacific Orange	HI30	Invitrogen†
	N/A	NA	NA	CD45 - FITC	HI30	BD Biosciences ¹
	NA	NA	NA	CD45-PerCP Cy5.5	2D1	BD Biosciences*
	NA	NA	NA	CD117-PE	YB5.B8	BD Biosciences*
	NA	NA	NA	CD117-PE-Cy7	104D2D1	Beckman Coulter‡
	NA	NA	NA	CD117-APC	YB5.B8	BD Biosciences*
Validation of microarray results	<i>CD2</i>	NS	NS	CD2-FITC	S5.2	BD Biosciences*
	<i>CD4</i>	5.9	4.3	CD4-Pacific Blue	RPA-T4	BD Biosciences*
	<i>CD33</i>	2.0	NS	CD33-APC	P67,6	BD Biosciences*
	<i>CD52</i>	NS	4.5	CD52-PE	CF1D12	Invitrogen†
	<i>CR2</i>	3.0	10.3	CD21-APC	B-ly4	BD Biosciences*
	<i>FCGR2B</i>	NS	2.2	CD32-PE	AT-10	Cytognos§
	<i>HLA-DRB5</i>	NS	2.2	HLA-DR-FITC	L234	BD Biosciences*
	<i>IL2RA</i>	13.4	19.7	CD25-PE	2A3	BD Biosciences*
	<i>IL3RA</i>	NS	2.4	CD123-PE	9F5	BD Biosciences*
	<i>LAIR1</i>	2.9	3.1	CD305-PE	DX26	BD Biosciences*

APC, Allophycocyanin; FC, fold change; FITC, fluorescein isothiocyanate; NA, not applicable; NS, not significantly deregulated; PE, phycoerythrin; PerCP, peridinin-chlorophyll-protein complex.

*Becton/Dickinson Biosciences, San Jose, Calif.

†Invitrogen, Carlsbad, Calif.

‡Beckman-Coulter, Miami, Fla.

§Cytognos, Salamanca, Spain.

TABLE E2. GEP score for the discrimination between patients with ISM and those with ASM: performance of individual (normalized) genes and the overall score and cutoff values, as assessed by using ROC curve analysis

Gene/parameter	AUC	P value	Cutoff	Sensitivity	Specificity
<i>CCL23</i>	0.93	<.001	$\geq 5.47^*$	83.3%	92.9%
<i>IL13</i>	0.89	.001	$\geq 2.32^*$	75.0%	92.9%
<i>IL1B</i>	0.87	.001	$\geq 2.19^*$	75.0%	92.9%
<i>CR2</i>	0.80	.009	$\geq 3.89^*$	66.7%	85.7%
Score	0.95	<.001	≥ 3	91.7%	100.0%

AUC, Area under the curve.

*Normalized expression is shown by a corrected fold increase and calculated as the ratio between the fold change (given by the ratio between the gene expression of each patient divided by the mean expression value for the control samples) of the target gene and that of the *GAPDH* housekeeping gene.