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Activation of the Janus kinase/signal transducer and activator of transcription pathway in multiple myeloma is not related to point mutations in kinase and pseudokinase domains of *JAK1*

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Abstract

Considering the recent impact of tyrosine kinase inhibitors in the treatment of myeloproliferative disorders carrying a recurrent *JAK2* mutation not identified in multiple myeloma (MM), this study aimed to search for mutations in kinase and pseudokinase domains of the *JAK1* gene in an attempt to define any critical and recurring change that can be used as a therapeutic target. We obtained CD138 + purified cells from 27 bone marrow aspirates of untreated MM, four normal controls and four MM cell lines. After amplification of kinase and pseudokinase domains of *JAK1* in cDNA samples, the fragments were automatically sequenced. Seventy-eight percent of MM cases showed at least one polymorphism, all being synonymous single nucleotide polymorphisms (SNPs), with allele frequencies consistent with previous studies in normal European, African American and Asian populations. The four cell lines also showed only synonymous SNPs. Mutations in the kinase and pseudokinase domains of the *JAK1* gene do not seem to be important for activation of the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway because we were not able to find any recurrent mutation in a case series of 27 patients and four MM cell lines.

Keywords: Multyple myeloma, JAK/STAT, *JAK1*, mutation

Introduction

Considering that the pathogenesis of multiple myeloma (MM) reflects processes related to increased cell survival and proliferation due to aberrant gene expression in the tumor cells, one area of particular interest has been the study of transcriptional factors that are activated inappropriately in a myeloma cell, in an attempt to find new therapeutic targets for this chronic but still incurable disease. Among them, the Janus kinase/signal transducer and activator of transcription (JAK/STAT) pathway can be persistently activated in patients with MM due to constant stimulation of this pathway by interleukin-6 (IL-6), produced by tumor cells and their microenvironment [1].

The JAK family is composed of proteins JAK1, JAK2, JAK3 and TYK2, with structural and functional homology, and plays an important role in the proliferation, differentiation and apoptosis of normal and tumor cells [2]. Translocations and mutations in *JAK* genes, which lead to constitutive activation of JAK proteins, are associated with various hematological malignancies, including myeloproliferative disorders (*JAK2*), acute lymphoblastic leukemia (*JAK2*), acute myeloid leukemia (*JAK1*, *JAK2*), acute megakaryoblastic leukemia (*JAK2*, *JAK3*) and T-cell precursor acute lymphoblastic leukemia (*JAK1*) [3–6], suggesting the importance of JAK/STAT as a potential target for therapies for these diseases. So far, only one study in MM has analyzed the presence of a mutation in *JAK2* Val617Phe, initially reported in patients with myeloproliferative disorders, but with negative results [7]. To date, we have not found any study exploring *JAK1* mutations in MM.

Most mutations in *JAK1* are point mutations affecting the kinase domain [8]. Different mutants of JAK1 protein activate distinct cytokine receptors, indicating specificity in activation of the signaling pathway based on the type of mutation in *JAK1* [9].

Considering that JAKs play a critical role in the signal transduction of many other cytokines, such as IL-6, which is involved in MM pathogenesis, blockade of JAK activation should down-regulate the supportive effects of aberrant JAK signaling in myeloma cells. Pharmacological inhibition of JAKs may, therefore, be a promising therapeutic strategy for the treatment of myeloma [10]. Therefore, the aim of this study was to search for point mutations in the kinase and

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pseudokinase domains of the *JAK1* gene in an attempt to define any critical change that can be used as a therapeutic target for MM.

Patients and methods

Twenty-seven bone marrow aspirates from untreated patients with MM, four normal controls (a peripheral blood sample and three reactive palatine tonsils) and four MM cell lines (U266, SKO-007, SKMM-2, RPMI8266) were analyzed. Patients were diagnosed according to the MM International Working Group Criteria [11], and tumor stage was classified according to Durie–Salmon and the International Staging System (ISS) [12]. Written informed consent was obtained from all patients and controls, and the study was approved by the Ethical Committee of our institution (CEP approval number is 1155/09).

Expression of *JAK1* and *TYK2* were evaluated by the real-time polymerase chain reaction (PCR) detection system ABI PRISM® Sequence 7500 (Applied Biosystems, Foster City, CA) in four MM cell lines. The constitutive gene *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) was chosen as endogenous control. SYBR Master Mix (Applied Biosystems) was used to perform quantitative PCR (qPCR) and all samples were analyzed in triplicate. Positive and negative controls were analyzed in parallel to verify the fidelity of amplification. The TaqMan® Array Human JAK/STAT Pathway, Fast 96-Well Plate was used to assess U266 and SKO-007 JAK/STAT related gene expression. Data were evaluated by DataAssist v3.0. Each gene was considered differentially expressed when it was three times higher in a cell line over another.

CD138+ purified cells from bone marrow MM aspirates and tonsils were obtained using microbeads conjugated to the monoclonal antibody CD138 (syndecan-1) (MACS, Magnetic Cell Sorting of Human Cells; Miltenyi Biotec, Germany). RNA was obtained using TRIzol reagent (Invitrogen, Frederick, MD) following the manufacturer's instructions, and cDNA was synthesized using the Superscript II kit (Invitrogen, Carlsbad, CA). To detect mutations in the *JAK1* gene, primers were designed covering the kinase (exons 12–18) and pseudokinase (exons 19–24) domains. The same was performed for the *TYK2* gene. After amplification, fragments were sequenced using forward and reverse primers (Table I). The sequencing reactions were performed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Waltham, MA) using an ABI PRISM 377 DNA Sequencer (Perkin Elmer). Sequencing product reading was performed by an ABI 3130 Genetic Analyzer (Applied Biosystems) and the software BioEdit Sequence Alignment Editor V7.0, the tool used to align the sequences [13]. To determine the single nucleotide polymorphisms (SNPs) found in kinase and pseudokinase domains of *JAK1* gene that had already been described, a search was made in the Database of Short Genetic Variations of NCBI (http://www.ncbi.nlm.nih.gov/snp). For each SNP, the genotype distribution and the allele frequency are described.

Table I. Direct and reverse primers for *JAK1* and *TYK2* kinase and pseudokinase domain amplification.

		Size of amplified fragment (bp)
Exons *JAK1*		
*JAK1*A F	CCATGAGCCAGCTGAGTTTCGAT	779
*JAK1*A R	CATGCAGCGGGTCATGAGATCA	
*JAK1*B F	TGACCTCATGACCCGCTGCATG	991
*JAK1*B R	GGTTCTGAAAGCTTGTCCGATTGGA	
*JAK1*C F	TGGAGCTTTGGAACCACGCTCTG	521
*JAK1*C R	TTTCCTCCGTCTTCTGTGCAGATTC	
Exons *TYK2*		
*TYK2*A F	CTGCGTCGCTGTTGCCTGC	731
*TYK2*A R	CTGTTGGCCCCACCTGGTA	
*TYK2*B F	AGGGAGGAGCGGGTGGAGA	745
*TYK2*B R	GCGCGTGCAGATAGGCCATG	
*TYK2*C F	TGGGCAGCCTCCGAGACTAC	708
*TYK2*C R	CCTCTTGGTTTCATCCTGGAGC	

Results

Figures 1(A) and 1(B) show relative expressions of *JAK1* and *TYK2* in MM cell lines, using *GAPDH* as housekeeping gene and U226 as reference. U266, SKO-007, SKMM-2 and RPMI8266 showed *JAK1* and *TYK2* expression. These results plus PCR array findings confirmed previous literature [14,15] reports about JAK/STAT deregulation in MM, and were our starting point to assume that they could be explored as candidates for recurrent mutations in MM. Unfortunately we did not have enough RNA to explore *JAK1* and *TYK2* expression in patient samples.

Twenty-one of 27 (78%) cases of MM had at least one polymorphism, all synonymous SNPs, with allele frequencies consistent with studies in normal European, African American and Asian populations (National Center for Biotechnology Information [NCBI]; http://www.ncbi.nlm.nih.gov/snp), as follows: at codon 733 (CCA> CCG), A (0.52) and G (0.48); at codon 683 (AGC> AGT), C (0.89) and T (0.11); at codon 1032 (AAG> AAA), G (0.89) and A (0.11); at codon 659 (CGC> CGT), C (0.91) and T (0.09); at codon 699 (GCC> GCG), C (0.93) and G (0.07) (Table II). Except for the SNP at codon 733 (CCA> CCG), homozygous carriers of the minor allele are absent or very rare in both cases of MM and a normal population (Table II). The clinical features of 27 cases of MM are described in Table III.

The four cell lines also showed only synonymous SNPs: 4/4 at codon 683 (AGC> AGT) and 1/4 at codon 733 (CCA> CCG). Among the four controls, one had a synonymous SNP at codon 733 (CCA> CCG), one at codon 683 (AGC> AGT) and one at codon 1032 (AAG> AAA).

Of the four cell lines evaluated for *TYK2* mutations, 100% sequencing showed good quality, and no change was found in these samples. Three palatine tonsil samples and one peripheral blood sample were used as negative controls, and no change was found in these samples either. Of the 27 cases evaluated, only 10 (37.0%) had good quality sequencing for the kinase and pseudokinase domains of the *TYK2* gene (fragments *TYK2*A, *TYK2*B and *TYK2*C), and no change was found in these samples. Amplification of the *TYK2*A fragment was only possible in 11 of 27 cases (40.8%), and none had abnormalities. Amplification of the *TYK2*B fragment was

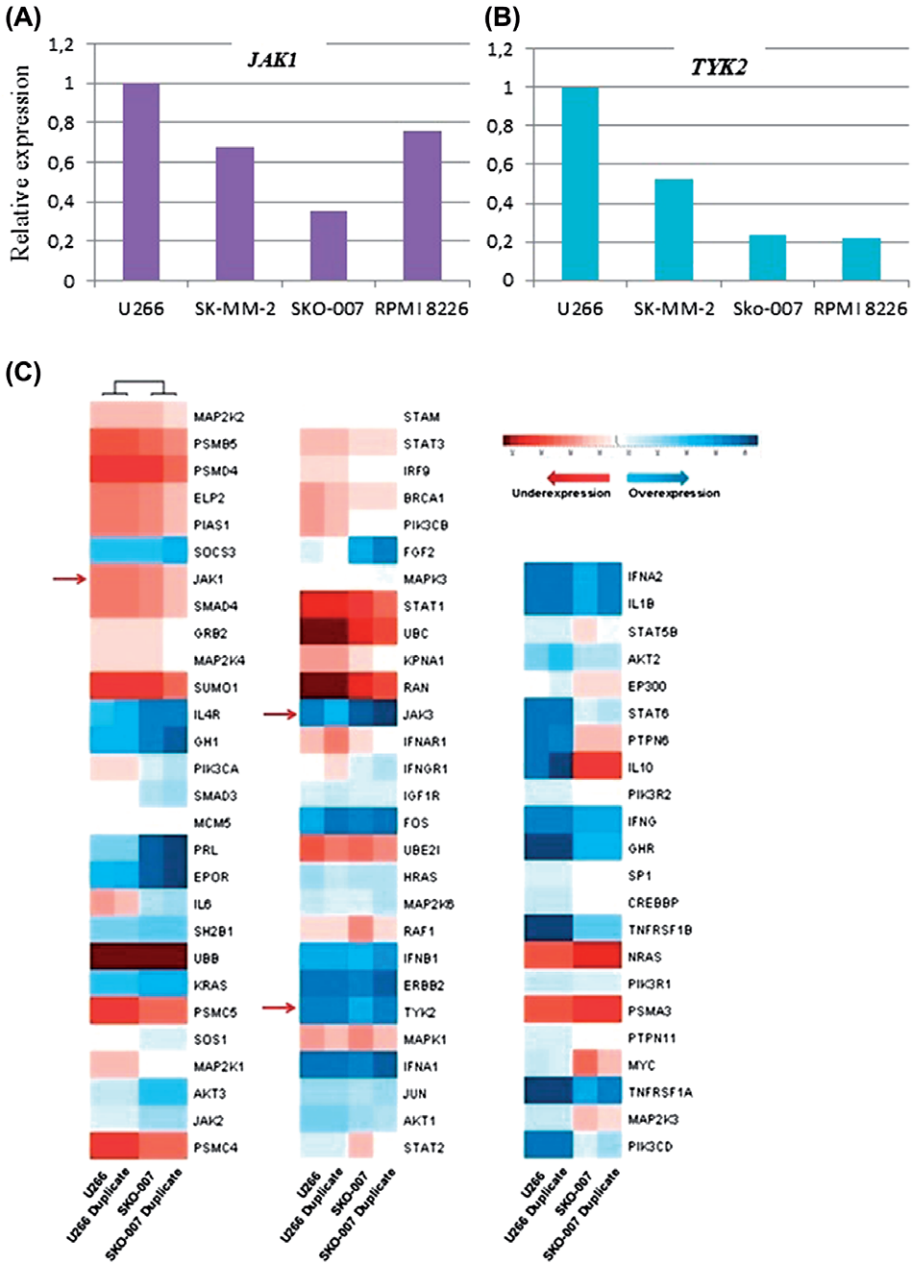

Figure 1. (A) Relative expression of *JAK1* in MM cell lines, using *GAPDH* as housekeeping gene and U226 as reference. (B) Relative expression of *TYK2* in MM cell lines, using *GAPDH* as housekeeping gene and U226 as reference. (C) Heat-map showing gene expression of the JAK/STAT pathway in U266 and SKO-007 cell lines.

possible in 23 of 27 cases (85.2%), and none had abnormalities. Amplification of the fragment *TYK2*C was possible in 25 of 27 cases (92.6%), and none had abnormalities.

Discussion

Mutations in the kinase and pseudokinase domains of the gene *JAK1* do not seem to be important for activation of the JAK/STAT pathway because we were not able to find any recurrent mutation in a case series of 27 patients and four MM cell lines. Other mechanisms, in addition to stimulation by IL-6, may be involved in this pathway activation in MM, and should be investigated.

It is not possible to completely rule out that chance might have played a role in determining our negative results. However, it is very unlikely that a mutation with an important role in multiple myeloma was not identified in our series of 27 cases and four MM cell lines.

Other mutations have been found in studies in patients with MM, but none in the *JAK1* gene, confirming our results. Recently, Chapman *et al.* [16] performed whole-genome sequencing in 23 patients and whole-exome sequencing in 16 patients, with one patient analyzed by both approaches. Their findings were related to mutations in genes involved in RNA processing, protein translation and unfolded protein response, in about half of the patients. These mutations can be important in MM due to the tremendous rate of immunoglobulin production by MM tumor cells [17–19]. Therefore, this study corroborates the success of the drug bortezomib (a proteasome inhibitor) that shows

Table II. Genotype distribution and allelic frequency of single nucleotide polymorphisms (SNPs) found in *JAK1* gene: comparison with other populations.

Study population	Exon	Codon	Base exchange	Amino acid	SNP ID	Sample	Genotype distribution (*n*)			Allelic frequency	
	14	659	CGC> CGT	Arg> Arg	rs17127063	Total	CC	CT	TT	C	T
Present study						27	23 (0.85)	3 (0.11)	1 (0.04)	0.91	0.09
European*						24	22 (0.92)	2 (0.08)	0	0.96	0.04
African American*						23	18 (0.78)	5 (0.22)	0	0.89	0.11
Asian*						23	23 (1.0)	0	0	1.00	0
	15	683	AGC> AGT	Ser> Ser	rs2230587	Total	CC	CT	TT	C	T
Present study						27	21 (0.78)	6 (0.22)	0	0.89	0.11
European†						24	20 (0.83)	4 (0.17)	0	0.92	0.08
African American†						23	16 (0.70)	6 (0.26)	1 (0.04)	0.82	0.17
Asian†						24	17 (0.71)	6 (0.25)	1 (0.04)	0.83	0.17
	15	699	GCC> GCG	Ala> Ala	rs3737139	Total	CC	CG	GG	C	G
Present study						27	23 (0.85)	4 (0.15)	0	0.93	0.07
European‡						89	80 (0.90)	9 (0.10)	0	0.95	0.05
African American‡						89	89 (1.00)	0	0	1.00	0
Asian‡						43	43 (1.00)	0	0	1.00	0
	16	733	CCA> CCG	Pro> Pro	rs2230588	Total	AA	AG	GG	A	G
Present study						27	10 (0.37)	8 (0.30)	9 (0.33)	0.52	0.48
European§						24	17 (0.71)	6 (0.25)	1 (0.04)	0.83	0.17
African American§						23	2 (0.09)	16 (0.70)	5 (0.22)	0.44	0.57
Asian§						24	9 (0.38)	12 (0.50)	3 (0.13)	0.63	0.38
	22	1032	AAG> AAA	Lys> Lys	rs12129819	Total	GG	GA	AA	G	A
Present study						27	22 (0.82)	4 (0.15)	1 (0.04)	0.89	0.11
European¶						24	19 (0.79)	5 (0.21)	0	0.90	0.10
African American¶						23	21 (0.91)	2 (0.09)	0	0.96	0.04
Asian¶						24	24 (1.00)	0	0	1.00	0

*NCBI Assay ID: ss552365572.
†NCBI Assay ID: ss5523155064.
‡NCBI Assay ID: ss5561710265.
§NCBI Assay ID: ss5523155047.
¶NCBI Assay ID: ss552424158.

remarkable activity in MM compared to other tumor types [20]. Chapman *et al.* [16] also observed 10 point mutations and four structural rearrangements affecting 11 nuclear factor-κB (NF-κB) pathway genes. Even though it is known that the NF-κB pathway is activated in MM, the process of this activation is still poorly understood [21,22].

Table III. Clinical features of 27 patients with MM.

Case/gender	Age	Isotype	Light chain	% Tumor cells	Stage	ISS
1. F	60	IgG	κ	80	IIIA	2
2. F	57	IgA	λ	90	IIIB	3
3. M	60	–	NA	92	IIA	NA
4. F	57	–	κ	NA	IIIA	NA
5. M	80	IgG	κ	90	IIIA	3
6. M	30	–	λ	95	IIIA	2
7. M	51	–	κ	90	IIIA	2
8. M	62	IgG	λ	80	IIIA	2
9. M	55	IgG	κ	70	IIIB	2
10. M	62	IgG	κ	30	IIIA	NA
11. M	63	IgA	λ	90	IIIB	2
12. M	65	IgA	κ	70	IIIB	3
13. M	62	IgG	λ	80	IIIA	NA
14. F	78	IgG	λ	60	IIIA	3
15. M	66	IgG	κ	80	IIIA	2
16. M	54	–	λ	85	IIIB	3
17. M	76	IgG	κ	21	IIIA	2
18. F	79	–	κ	100	IIIB	3
19. F	45	IgG	λ	90	IIIA	3
20. F	51	IgG	κ	42	IIIA	2
21. M	60	IgG	λ	80	IIIB	3
22. M	64	–	κ	90	IIIA	3
23. F	78	IgM	λ	20	IIIA	2
24. M	58	IgG	κ	90	IIIB	2
25. M	82	IgA	κ	15	IIIA	2
26. M	59	IgG	κ	30	IIIB	3
27. M	80	IgA	λ	95	IIIB	3

Ig, immunoglobulin; ISS, International Staging System; NA, not available.

Although mutations in genes of the JAK/STAT pathway have not been demonstrated by previous studies, there is still a common sense that this particular pathway is relevant to the pathogenesis of MM. Transcriptional factors may be constantly activated, not because of gene mutations, but due to other molecular causes, such as constant stimulation by cytokines or other molecular defects. These transcription factors can serve as convergence points of multiple signaling pathways, and are involved in the regulation of various genes that are responsible for the malignant behavior of a myeloma cell [23].

One transcriptional factor that appears to be of relevance for MM as an oncogenic regulator is STAT3, a member of the STAT family of proteins, which transmits signals when cytokines and growth factors bind to the receptors located in the cell surface [24]. Constitutive expression of STAT3 has been seen frequently in patients with MM and lymphoma [25]. Some studies involving synthetic molecules inhibiting JAK showed suppression of phosphorylation of STAT3, the transcription factor activated by IL-6, inducing apoptosis in MM cell lines [14,15,26,27].

Li *et al.* [10] studied the effects of INCB 16562, a JAK1/2 selective inhibitor, in myeloma cell lines. They demonstrated the effects of the JAK1/2 inhibitor in tissue models and *in vivo*. This compound was able to potentiate the effects of numerous therapeutics by diminishing the protective effects of IL-6. Furthermore, when combined with melphalan or bortezomib, this compound inhibited, almost completely, tumor growth. These studies suggest that, while there is not a mutation responsible for constitutive activation of this pathway (as in myeloproliferative disorders), inhibition of

the JAK/STAT pathway may still be a potential target for the treatment of patients with MM.

Results regarding the *TYK2* gene are not highlighted here because some samples were in poor condition for kinase and pseudokinase domain sequencing, and we were not able to evaluate the three fragments of this gene in all 27 cases. Therefore, we cannot assume that no mutation in *TYK2* was present in our cases.

Potential conflict of interest: Disclosure forms provided by the authors are available with the full text of this article at www.informahealthcare.com/lal.

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