Analysis of Signal Transducer and Activator of Transcription 3 (Stat 3) Pathway in Multiple Myeloma

Stat 3 Activation and Cyclin D1 Dysregulation Are Mutually Exclusive Events

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The signal transducer and activator of transcription molecules (Stats) play key roles in cytokine-induced signal transduction. Recently, it was proposed that constitutively activated Stat 3 (Stat 3 phosphorylated) contributes to the pathogenesis of multiple myeloma (MM) by preventing apoptosis and inducing proliferation. The study aim was to investigate Stat 3 activation in a series of multiple myeloma (MM) cases and its effect on downstream targets such as the anti-apoptotic proteins Bcl-xL, Mcl-1, and Bcl-2, and the cell-cycle protein cyclin D1. Forty-eight cases of MM were analyzed. Immunohistochemistry was performed on paraffin sections using antibodies against cyclin D1, Bcl-2, Bcl-xL, Mcl-1, p21, Stat 3, and Stat 3 phosphorylated (P). Their specificity was corroborated by Western blot analysis using eight human MM cell lines as control. The proliferation rate was assessed with the antibody MiB1. In addition, the mRNA levels of cyclin D1 and Stat 3 were determined by quantitative real-time reverse transcriptase-polymerase chain reaction of paraffin-embedded microdissected tissue. Three different groups determined by the expression of Stat 3P and cyclin D1 (protein and mRNA) were identified: group 1, Stat 3-activated (23 cases, 48%). All cases revealed nuclear expression of Stat 3P. No elevation of Stat 3 mRNA was identified in any of the cases. Three cases in this group showed intermediate to low cyclin D1 protein and mRNA expression. Group 2 included 15 (31%) cases with cyclin D1 staining and lack of Stat 3P. All cases showed intermediate to high levels of cyclin D1 mRNA expression. Group 3 included 10 (21%) cases with no expression of either cyclin D1 or Stat 3P. High levels of anti-apoptotic proteins Bcl-xL and Mcl-1 were identified

in 89% and 100% of all cases, respectively. In contrast to Bcl-xL and Mcl-1, the expression of Bcl-2 showed an inverse correlation with proliferation rate (P : 0.0003). No significant differences were found between the three groups in terms of proliferation rate or expression of anti-apoptotic proteins. However, cyclin D1+ cases were always well differentiated and were more likely to show a lymphoplasmacytoid differentiation (χ^2 -square = 9.55). Overall, constitutive activation of Stat 3 was found in almost half (48%) of the investigated MM cases. However, this does not seem to have a major impact on the expression of anti-apoptotic proteins and proliferation. We showed that cyclin D1 overexpression and Stat 3 activation are, mutually exclusive events in MM (P = 0.0066). The universal expression of Mcl-1, independent of activated Stat 3, suggests that its expression is constitutive and that it might play an important role in the pathogenesis of MM. (*Am J Pathol* 2003, 162:1449–1461)

Signal transducer and activator of transcription (Stat) 3 is a cytoplasmic latent transcription factor that becomes activated by phosphorylation, typically in response to extracellular ligands such as interleukin (IL)-6, platelet-derived growth factor, or epidermal growth factor.¹ Specifically, IL-6 binds to its α chain receptor and induces homodimerization of gp 130 and activation of the intracytoplasmic Janus family of tyrosine kinases (Jaks), with downstream signaling via the Stat- or Ras-dependent mitogen-activated protein kinase cascades. Once phosphorylated by Janus kinases, Stat 3 dimerizes and translocates to the nucleus, where it activates the transcription

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of target genes. Stat 3 activation has been implicated in the regulation of cell proliferation, differentiation, and apoptosis.² The importance of Stat 3 gene is highlighted by the fact that its disruption in animal models causes embryonic lethality.³ In the last years, several studies have shown that tumor cell lines and samples derived from human cancers, including breast, hematopoietic, head and neck, lung, kidney, prostate, and ovarian cancers frequently express activated or phosphorylated Stat 3 (Stat 3P),⁴ suggesting that Stat 3 plays a critical role in regulating fundamental processes associated with malignant transformation and cell survival.⁵ Accordingly, recent *in vitro* data demonstrated that a constitutively active form of Stat 3 was sufficient to produce anchorage-independent cell proliferation and tumor formation in nude mice, thus emphasizing its oncogenic potential.⁶ However, the biological mechanisms by which Stat 3 contributes to oncogenesis are not completely understood. Critical Stat 3-regulated genes proposed to be involved in the oncogenic process are cyclin D1, *c-myc*, and Bcl-xL, whose mRNA levels were found threefold to fivefold up-regulated as a consequence of Stat 3 activation.⁶

Recently, Catlett-Falcone and colleagues,⁷ reported that constitutively activated Stat 3 is expressed in U266 myeloma cells and in the neoplastic cells of most patients with multiple myeloma (MM), in a third of them at very high levels. They presented evidence that constitutive Stat 3P induces Bcl-xL expression and confers resistance to Fas-induced apoptosis in U266 cells. Another anti-apoptotic member of the Bcl-2 family induced by IL-6 in a Stat 3-dependent way, myeloid cell factor 1 (Mcl-1), also has been reported to be a critical survival factor for MM.^{8–10} The deregulated expression of Bcl-xL and Mcl-1 in MM is of particular interest because MM is a B-cell neoplasia characterized by accumulation of slowly proliferating malignant plasma cells, and the up-regulation of anti-apoptotic genes is thought to play an important role in the pathogenesis of this disorder.¹¹

However, activated Stat 3 induces also genes that are involved in the control of cell cycle progression and proliferation such as cyclin D1 and *c-myc*. Cyclin D1 protein is overexpressed in 25 to 30% of the MM cases, presumably as a consequence of the t(11;14).^{12–15} However, the t(11;14) is identified in only 4 to 10% of bone marrow specimens when examined by conventional cytogenetics, and in 15 to 20% using fluorescent *in situ* hybridization.^{12,16} Therefore, in a proportion of MM cases with dysregulated cyclin D1 no apparent molecular abnormalities of the *bcl-1* locus are identified.^{12–15} The reason(s) for cyclin D1 dysregulation in these latter cases are unknown. Because cyclin D1 is one of the main target genes of Stat 3, we wished to study the possible interaction of activated Stat 3 and cyclin D1 dysregulation in MM. For these reasons we analyzed in a series of primary MM: 1) the frequency of constitutively activated Stat 3, 2) the level of cyclin D1 mRNA and protein expression, and 3) the downstream effects of activated Stat 3 on proliferation and induced anti-apoptosis.

Materials and Methods

Tissue Samples

Forty-eight formalin-fixed, paraffin-embedded and non-decalcified blocks of tissue specimens obtained from lytic bone lesions of MM diagnosed between 1991 and 2001 were selected from the files of the Institute of Pathology, Technical University of Munich, Munich, Germany. Most of the material was obtained during surgery, and contained a high percentage of tumor cells with few bone trabeculae. Clinical information was obtained from the patients' medical records. Hematoxylin and eosin-stained slides and immunoperoxidase studies were reviewed in all cases by three of the authors (LQ-M, MK, and FF). The cases were graded according to the histological grading criteria described by Bartl and colleagues¹⁷ The clinical staging was done according to Durie and Salmon.¹⁸ Some of the cases have been reported previously as part of another study.¹⁹

Immunohistochemistry

Immunohistochemistry was performed on an automated immunostainer (Ventana Medical Systems, Inc., Tucson, AZ) according to the company's protocols, with slight modifications. After deparaffinization and rehydration, the slides were placed in a microwave pressure cooker in 0.01 mol/L citrate buffer, pH 6.0, containing 0.1% Tween 20, and heated in a microwave oven at maximum power for 30 minutes. The antibody panel used included cyclin D1 (clone P2D11F11; Novocastra, Newcastle, UK), Stat3 (BD Transduction Laboratories; San Diego, CA), Stat3 phosphorylated (P) (New England Biolabs Inc., Beverly, MA), p21 (BD Transduction Laboratories), Bcl-2 (DAKO, Glostrup, Denmark), Bcl-xL (BD Transduction Laboratories), Mcl-1 (Chemicon, Temecula, CA), CD20 (clone L26, DAKO), and p27KIP1 (BD Transduction Laboratories). The proliferation rate was assessed with the monoclonal antibody against the Ki67 antigen (clone MiB-1, DAKO). Appropriate positive controls were used to confirm the adequacy of the staining. The Stat 3 antibody from Transduction Laboratories recognizes both the latent (cytoplasmic) and the activated (nuclear) form of Stat 3, whereas the Stat 3P antibody recognizes exclusively the nuclear or activated form of Stat 3. As control for the anti-Stat 3 antibodies, cell blocks from two MM cell lines were used. To assure the staining quality of cyclin D1, a cyclin D1-positive mantle cell lymphoma carrying the t(11;14) translocation was included in every run. Because p21 expression is found only in isolated lymphocytes, any staining >10% was considered positive. A grid ocular objective was used to count 300 cells over three high-power fields (×40) and the percentage of positive cells was reported as 0 to 100%.

Cell Lines

In addition to the primary cases, eight human myeloma cell lines (KMM1, OPM2, U266, KMS5, KMS11, KMS12,

Table 1. Sequence of TaqMan Primers and Probes Used in This Study

Oligonucleotide	Location	Sequence	Size PCR product
Stat 3 FP	2284 F	GCC AGA GAG CCA GGA GCA	74 bp
Stat 3 RP	2358 R	ACA CAG ATA AAC TTG GTC TTC AGG TAT G	
Stat 3 probe	2305 T	TGA AGC TGA CCC AGG TAG CGC TGC	
CycD1 FP	307 F	5'-CCGTCCATGCGGAAGATC-3'	69 bp
CycD1 RP	376 R	5'-CCTCCTCCTCGCACTTCTGT-3'	
CycD1 probe	331 T	5'-CTCGCAGACCTCCAGCATCCAGGT-3'	
TBP FP	645 F	5'-GCCCCAAACGCCGAATAT-3'	72 bp
TBP RP	717 R	5'-CCGTGGTTCGTGGCTCTCT-3'	
TBP probe	664 T	5'-ATCCCAAGCGGTTTGCTGCGG-3'	

KMS18, KMS20) were used in this study. A mantle cell lymphoma cell line, Granta 519, was used as control. All cell lines were cultured in RPMI 1640 medium supplemented with 15% fetal calf serum, 2 mmol/L glutamine, 10 U/ml penicillin, and streptomycin (Gibco, Life Technologies). Protein concentration was determined by the BCA protein assay reagent kit (Pierce, Rockford, IL).

Preparation of Cell Line Blocks

Tumor cell lines were grown in RPMI 1640 supplemented with 15% fetal calf serum. The cells were harvested during their exponential growth phase, centrifuged at $550 \times g$ for 5 minutes, and the supernatant removed. The cell pellet was washed once and resuspended in three drops of human plasma to which three drops of thrombin were added. The resulting clot was fixed in 10% formalin and paraffin-embedded.

Western Blot Analysis

A total of 60 μ g of protein extracts was separated by 7.5% and/or 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to pure nitrocellulose immobilization membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were blocked for 1 hour in 5% nonfat dry milk and incubated with the primary antibody for 1 hour. Subsequently, membranes were washed five times for 5 minutes each in a wash buffer (10 mmol/L Tris, pH 7.6, 100 mmol/L NaCl, and 0.1% Tween) and incubated with a biotinylated secondary antibody for 1 hour. Membranes were washed five times in the same wash buffer and detection was performed by chemiluminescence with the ECL detection system (Amersham Pharmacia Biotech) for 2 to 15 minutes, and then the membranes were exposed to a Kodak X-OMAT AR film. All assays were repeated several times and gave similar results.

IL-6 Enzyme-Linked Immunosorbent Assay

IL-6 was measured in the supernatant of the cell cultures, 5 days from splitting during their exponential growth phase using the Quantikine human IL-6 kit from R&D Systems (Minneapolis, MN). The enzyme-linked immunosorbent assay was performed according to the recommendations of the manufacturer without modifications.

The optical density was measured with the microplate reader DigiScan from Asys Hithec, Germany. The used wavelength was 450 nm with a wavelength correction at 570 nm as recommended. The standard curve was created by reducing the data using a computer software (Mikrotek Labor Systems GmbH, Overath, Germany) capable to generate a four-parameter logistic (4-PL) curve fit as recommended.

Real-Time Quantitative Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)

Tissue preparation, microdissection of pure tumor cell populations, and RNA extraction from formalin-fixed tissues for real-time quantitative RT-PCR were performed as described previously.²⁰ Real-time RT-PCR reactions were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Intron-spanning primers and probes for cyclin D1, Stat 3, and TATA box-binding protein (TBP) as housekeeping gene control were designed using Primer Express software (Applied Biosystems) (Table 1).

Ten μ l of RNA extracted from microdissected cells was transcribed into cDNA using Superscript II reverse transcriptase (Invitrogen) and 250 ng of random hexamers (Roche, Penzberg, Germany) following the manufacturer's recommendations in a final volume of 20 μ l. PCR reactions were performed in at least two replicates and were performed with the TaqMan Universal PCR Master Mix (Applied Biosystems) using 6 μ l of diluted cDNA, 200 nmol/L of the labeled probe, and 300 nmol/L of primers (except cyclin D1-307 sense that was used at 900 nmol/L) in a 30- μ l final reaction mixture. After initial incubation at 50°C for 2 minutes and 95°C for 10 minutes, samples were amplified for 50 cycles of 95°C for 15 seconds, followed by 60°C for 1 minute.

Amounts of cyclin D1, Stat 3, and TBP mRNAs were calculated using linear regression analysis from an external standard curve generated from Granta 519 RNA. To determine the threshold value for altered cyclin D1 and Stat 3 expression in MM, the mean values of cyclin D1/TBP and Stat 3/TBP ratios in reactive tissues from lymph nodes and bone marrow were evaluated. The mean cyclin D1/TBP ratio in reactive tissues was 0.84 (range, 0.31 to 1.38). Cyclin D1/TBP ratios greater than the value of 2.33 (mean + 5 SD) were arbitrarily considered to represent increased cyclin D1 expression. The Stat3/TBP ratio in reactive tissues

Table 2. Clinicopathological Features of 48 MM Patients Included in the Study

Case	Age	Sex	MG	Histology	Stage	Treatment	Follow-up
Group 1. Stat3P+							
1	56	F	***	I-LP	Ia	RT	DwD 28 months
2	69	M	G, λ	I	Ia	CT	ANED 26 months
3	74	F	κ	I	IIla	***	DwD 28 months
4	75	F	κ	I	IIla	RT + CT	DwD 48 months
5	64	F	G, λ	I	IIla	RT + CT	AwD 30 months
6	52	M	G, λ	I	Ia	RT	AwD 22 months
7	64	M	G, κ	II	IIla	RT	DwD 83 months
8	79	F	***	I	***	***	LFU
9	54	M	λ	I-LP	IIla	CT	ANED 26 months
10	63	M	G, λ	I	Ia	RT + CT	DwD 84 months
11	72	F	***	II	Ia	***	LFU
12	75	M	***	II	***	***	LFU
13*	63	F	A, λ	I	IIla	RT + CT	AwR 96 months
14	64	M	G, λ	I	IIla	CT	LFU
15	55	F	λ	I	IIla	RT + CT	AwD 4 months
16	74	F	G, κ	I	IIla	RT + CT	AwD 6 months
17	57	M	***	I	***	***	LFU
18	67	M	G, κ	I-LP	Ia		AwD 36 months
19	32	M	G, κ	I	Ia	RT + CT + T	AwD 53 months
20	74	F	G, κ	I	IIla	RT + CT	DwD 12 months
21	62	F	M, λ	II	Ia	RT	AwD 12 months
22	60	F	G, λ	II	IIla	CT	AwD 22 months
23*	76	M	A, λ	II	IIlb	RT + CT	AwR 51 months
Group 2. Cyclin D1 +							
24	59	M	***	I-LP	***	***	LFU
25	59	M	κ	I-LP	IIlb	CT	DwD 29 months
26	76	M	κ	I-LP	Ia	RT	AwD 15 months
27	76	F	λ	I	IIla	RT + CT	DwD 11 months
28	58	M	G, κ	I-LP	IIla	RT + CT	AwD 87 months
29	56	F	G, κ	I	IIla	RT + CT	AwD 91 months
30	78	F	G, κ	I	IIlb	RT + CT	DwD 48 months
31	64	M	G, κ	I-LP	Ia	***	AwD 1 month
32	81	M	λ	I-LP	IIla	RT + CT	DwD 10 months
33	40	M	G, κ	I	IIlb	CT + T	AwD 36 months
34	61	F	κ	I	IIla	RT	AwD 3 months
35	69	F	G, κ	I	Ia	RT	AwD 25 months
36	78	M	***	I	***	***	LFU
37	66	M	G, κ	I-LP	Ia	CT	AwD 41 months
38	59	M	G, κ	I	Ia	RT	AwR 75 months
Group 3. Stat3P- and cyclin D1-negative							
39	73	M	G, κ	I	IIla	RT + CT	AwD 54 months
40	76	M	G, κ	II	IIla	***	DwD 23 months
41	63	F	G, κ	II	IIla	RT	LFU
42	48	F	λ	II	IIla	RT + CT + T	AwD 46 months
43	69	F	G, κ	I	IIla	RT + CT	LFU
44	71	F	G, κ	I	IIlb	RT + CT	AwD 29 months
45	47	F	κ	I	IIla	RT + CT	DwD 67 months
46	61	M	***	I	Ia	***	LFU
47	60	F	G, κ	I	IIla	RT + CT	AwD 27 months
48	68	M	***	I	***	***	LFU

MG, monoclonal gammopathy; AwD, alive with disease; DwD, died with disease; ANED, alive no evidence of disease; LFU, lost to follow-up; AwR, alive with recurrence; CT, chemotherapy; RT, radiotherapy; ***, data are not available; *, only the biopsy at relapse was available; T, autologous stem-cell bone marrow transplant; LP, lymphoplasmacytoid morphology.

from BM and LN was also determined (mean, 1.27; range, 1.15 to 1.38). Stat3/TBP ratios below 1.79 (mean + 5 SD) were arbitrarily considered to represent normal Stat3 levels.

Statistical Analysis

The significance of the association of different clinicopathological parameters with the expression of Stat 3 and cyclin D1 or with the lack of expression of both proteins was assessed with the chi-square test. The association between Bcl-2 and proliferation rate was assessed using

the Mann-Whitney *U*-test and the Fisher's exact test. The association between the expression of Stat 3P and cyclin D1 was assessed using the Fisher's exact test.

Results

Patient Characteristics and Histological Findings

Clinical data and histology are summarized in Table 2. Of the 48 patients, 25 were male and 23 were female (M:F

Figure 1. Western blot analysis for Stat 3, Stat 3P, cyclin D1, and anti-apoptotic proteins Bcl-xL, Mcl-1, and Bcl-2 in human MM cell lines. Each lane contains 60 μ g of protein extract from the following cell lines: **lane 1**, Granta 519; **lane 2**, KMS-20; **lane 3**, U266; **lane 4**, KMS-18; **lane 5**, KMS-5; **lane 6**, KMS-11; **lane 7**, KMM1; **lane 8**, KMS-12; **lane 9**, OPM2. **Lane 1** (Granta 519, mantle cell lymphoma cell line) and **lane 8** (KMS12) represent the t(11;14) translocated cell lines. Expression of cyclin D1 is limited to these two cell lines. **Lane 3** (U266) represents the cell line with known constitutive activation of Stat 3. Western blot analysis with the N-terminal anti-Stat 3 antibody demonstrates both Stat 3 α and Stat 3 β (92 and 83 kd, respectively) in all cell lines. In contrast only KMS20 and U266 show strong positivity for Stat 3 phosphorylated. Bcl-xL and Mcl-1 expression is present in all cell lines with no apparent correlation between these proteins and the presence of phosphorylated Stat 3. Bcl-2 is negative in three of the cell lines (KMS20, KMS5, and KMM1). Note that the two cell lines with expression of cyclin D1 show high levels of Bcl-2.

ratio, 1:1), with a median age of 64 years (range, 32 to 81 years). In 12 patients the clinical information was not available. Histologically, 39 cases (81%) were composed of well-differentiated plasma cells (Bartl grade I) and 9 cases (19%) were moderately differentiated (Bartl grade II). Ten cases revealed lymphoplasmacytoid differentiation. None of the cases showed plasmablastic morphology.

Western Blot Analysis for Stat 3, Stat 3 Phosphorylated, Cyclin D1, Bcl-xL, Mcl-1, and Bcl-2 in Human MM Cell Lines

To validate the specificity of the Stat 3 antibodies used in this study, Western blot analysis was performed in well-characterized human MM cell lines (Figure 1). Western blot analysis with the N-terminal-specific anti-Stat 3 antibody that recognizes both Stat 3 α - and Stat 3 β -phosphorylated and -unphosphorylated proteins, demonstrated two specific protein bands of the expected size (92 and 83 kd, respectively) in all human MM cell lines. The mantle cell lymphoma cell line, Granta 519, used as control for cyclin D1 expression showed significantly weaker bands. In contrast, phosphorylated Stat 3 (Y705) (92 kd), which specifically recognizes Stat 3 phosphorylated at tyrosine 705, was strongly expressed only in U266 and KMS20 MM cell lines. As expected, cyclin D1 was expressed only in the two cell lines known to have a t(11;14); Granta 519 and the MM cell line KMS12. Bcl-xL and Mcl-1 were expressed in all cell lines and their levels of expression varied from one cell line to another (Figure 1). Five MM cell lines expressed high levels of Bcl-xL (U266, KMS18, KMS5, KMS11, and KMM1), and three expressed moderate levels (KMS20, KMS12, and OPM2).

The expression of Mcl-1 was more homogeneous and only U266 expressed higher levels when compared to the other cell lines. Bcl-2 was negative in three of the eight cell lines (KMS-20, KMS-5, and KMM1). Of note is that the two cell lines with t(11;14) and expression of cyclin D1 showed very high levels of Bcl-2.

IL-6 Expression in Human MM Cell Lines and Correlation with the Expression of Anti-Apoptotic Proteins

IL-6 was measured in the supernatant of all cell lines to identify those MM cell lines with autocrine production of IL-6. We found that KMS-20, U266, KMS18, KMS5, and KMM1 produce IL-6 in an autocrine manner, whereas the mantle cell lymphoma cell line Granta 519 and the MM cell lines KMS11, KMS12, and OPM2 are independent of IL-6 for their growth. Of note is that even though all cell lines expressed Stat 3 in steady-state, those cell lines with autocrine production of IL-6 had higher expression levels of Stat 3, with the exception of KMS18. However, the autocrine production of IL-6 in some cell lines did not universally result in the presence of Stat 3P, nor did it influence the expression of Bcl-xL, Mcl-1, and Bcl-2.

Immunohistochemical Findings in Primary MM Cases

The results of the immunohistochemical studies are summarized in Table 3. All cases were previously immunophenotyped for CD20 and CD138. All cases were positive for CD138. Four of the 48 cases (8.3%) showed expression of CD20 in a proportion of the tumor cells. To confirm the staining pattern of the Stat 3 antibodies in paraffin tissues, cell blocks from two MM cell lines were used. The KMS18 cell line showed a purely cytoplasmic staining with the Stat 3 antibody demonstrating the presence of steady-state Stat 3 (Figure 2A) and was negative for the Stat 3P antibody (Figure 2A, inset). In contrast the U266 cell line revealed both a cytoplasmic and nuclear positivity with the anti-Stat 3 antibody (Figure 2B, inset). The activation of Stat 3 in U266 cell line was confirmed with the Stat 3P-specific antibody, rendering a crisp nuclear staining (Figure 2B). For the purpose of this study, only cases with nuclear staining with both antibodies, indicating Stat 3 activation, were considered positive for Stat 3 (Figure 2, C and D). Cases with only cytoplasmic staining for Stat 3 but negativity for Stat 3P were considered Stat 3-negative (Figure 2, E and F). Following this definition, Stat 3 activation was observed in the majority of tumor cells in 23 of the 48 cases (48%). The staining varied from case to case; 13 cases showed strong (+++), 7 moderate (++), and 3 weak nuclear expression (+). Of the 25 cases considered negative for Stat 3P, 13 cases revealed cytoplasmic staining with the Stat 3 antibody, indicating the presence of steady-state Stat 3. Cyclin D1 expression was observed in 18 cases (37.5%).

Table 3. Immunohistochemical and Real-Time RT-PCR Analysis in 48 Cases of MM

Case	Immunohistochemistry							Real time RT-PCR	
	Stat 3P	cyc D1	MiB1	p21	bcl-2	bcl-xl	MCL-1	CycD1	Stat 3
Group 1. Stat 3P+									
1*	+	neg	6%	10%	pos	+++	+++	2.14	0.8
2	+++	neg	5%	<5%	pos	+++	+++	0.38	1.0
3	+++	neg	10%	30%	pos	++	+++	0.62	1.6
4	+++	neg	30%	7%	pos	+++	+++	0.14	0.5
5	++	neg	80%	53%	pos	neg	+++	0.26	0.9
6	++	neg	<5%	10%	neg	+++	+++	0.77	0.3
7	++	neg	25%	40%	neg	+++	+++	0.82	0.7
8	++	neg	24%	<5%	neg	++	+++	1.54	0.5
9	+	neg	10%	42%	pos	neg	+++	0.28	0.8
10	+++	neg	18%	14%	neg	+++	+++	0.26	0.5
11	++	neg	15%	5%	pos	++	+++	0.77	0.6
12	+++	neg	20%	10%	pos	+++	+++	0.67	0.4
13	+++	neg	5%	10%	pos	+++	+++	0.46	1.0
14	++	neg	80%	<5%	neg	+++	+++	0.14	0.4
15	+++	neg	<5%	<5%	pos	+++	+++	0.22	0.8
16†	+++	neg	20%	<5%	pos	+++	+++	7.78	0.7
17	+++	neg	64%	50%	neg	+++	+++	0.15	0.5
18	+++	++	30%	30%	neg	+++	+++	18.72	0.8
19	+++	+	<5%	<5%	pos	+++	+++	15.36	1.2
20	+++	++	80%	80%	neg	++	+++	3.04	0.7
21	+	neg	21%	<5%	neg	+++	+++	0.4	0.6
22	+++	neg	<5%	<5%	pos	++	+++	NE	NE
23	++	neg	<5%	25%	pos	+++	+++	2.0	1.2
Group 2. Cyclin D1+									
24	neg	+++	70%	41%	neg	neg	++	192.9	0.8
25	neg	+++	34%	<5%	pos	neg	++	145.0	0.2
26*	neg	+++	40%	<5%	pos	neg	++	101.38	0.5
27	neg	+++	<5%	<5%	pos	+++	+++	139.0	0.2
28	neg	+++	15%	10%	pos	+++	+++	408.0	0.7
29	neg	+++	18%	52%	pos	neg	++	163.0	0.2
30*	neg	+++	38%	38%	pos	+++	+++	425.0	0.6
31	neg	+++	<5%	30%	pos	+++	+++	666.25	NE
32	neg	+++	26%	100%	neg	+++	++	166.70	0.3
33	neg	+++	11%	8%	pos	+++	++	103.84	0.7
34	neg	+++	12%	90%	pos	+++	++	175.89	0.4
35	neg	++	12%	25%	pos	++	+++	15.34	2.8
36	neg	++	53%	<5%	neg	+++	+++	15.40	1.1
37	neg	+	15%	10%	neg	++	+++	12.42	2.5
38	neg	+	<5%	<5%	pos	+++	+++	10.49	1.6
Group 3. Stat 3P- and cyclin D1-negative									
39	neg	neg	15%	<5%	pos	++	+++	2.5	NE
40	neg	neg	70%	30%	neg	++	+++	0.13	1.2
41	neg	neg	34%	20%	neg	+++	+++	1.26	0.7
42	neg	neg	24%	<5%	pos	++	+++	1.55	NE
43*	neg	neg	<5%	10%	pos	++	+++	2.56	0.7
44	neg	neg	30%	<5%	neg	++	+++	0.87	0.2
45	neg	neg	<5%	<5%	pos	++	+++	1.01	1.1
46†	neg	neg	<5%	9%	pos	++	+++	6.6	0.1
47	neg	neg	<5%	10%	pos	+++	+++	3.63	1.3
48†	neg	neg	60%	7%	pos	+++	+++	3.28	0.4

Cyclin D1 +++, >80% tumor cells strongly positive; ++, 20 to 50% positive cells; +, 10 to 20% positive cells; Stat 3, +++, most tumor cells strongly positive; ++, moderately positive; +, weakly positive.

Bcl-xl and Mcl-1 were evaluated according to the intensity of the positivity, +++ strongly positive, ++ moderately positive. ND, not done.

*These cases were positive for CD20 in a proportion of the tumor cells.

†Rare plasma cells were positive for cyclin D1 by immunohistochemistry.

MiB1 >20% correlates with lack of expression of Bcl-2 ($P = 0.0001$).

In 11 of these 18 cases, homogenous, strong nuclear staining of cyclin D1 was observed in >80% of the tumor cells (+++) (Figure 3, A and B), in 4 cases in 20 to 50% of the tumor cells (++) (Figure 3, C and D), and in the remaining 3 cases, in 10 to 20% of the neoplastic cells (+) (Figure 3E). In contrast to cases with +++, the intensity of cyclin D1 staining in cases with ++ and + positivity was weak to moderate and varied from cell

to cell (Figure 3D). In three cases, unequivocal, rare plasma cells were positive for cyclin D1 (Figure 3F). These cases were interpreted as negative for cyclin D1. Mcl-1 was expressed in the cytoplasm of tumor cells in all cases (100%) (Figure 4A). However, in 7 of 18 cases with cyclin D1 expression, the intensity of the positivity was weaker (++) (Figure 4B). Bcl-2 was strongly positive in 32 of 48 cases (67%) (Figure 4, C

Figure 2. Immunohistochemical analysis of Stat 3 and Stat 3P. **A:** KMS18 cell line block used as control. The tumor cells show cytoplasmic positivity for Stat 3 without nuclear staining. **Inset:** Note that the cells are completely negative when stained with the Stat 3P antibody. **B:** U266 cell line block used as control. The tumor cells show a strong, crisp nuclear positivity for Stat 3P without cytoplasmic staining. **Inset:** Note that by immunostaining with the Stat 3 antibody, the cells show strong nuclear and cytoplasmic staining. **C-F:** Primary MM cases stained for Stat 3 and Stat 3P. **C:** Case 2, group 1. The tumor cells show cytoplasmic and nuclear positivity for Stat 3. **D:** The same case immunostained with the Stat 3P antibody reveals a strong nuclear expression confirming the presence of activated Stat 3. **E:** Case 36, group 2. The vast majority of tumor cells show cytoplasmic expression of Stat 3 indicating the presence of steady-state Stat 3. Note the lack of nuclear staining. **F:** The same case is negative for anti-Stat 3P. Note the positive nuclear staining of the endothelial cells used as internal control. Immunoperoxidase staining; original magnifications, $\times 400$.

and D). Bcl-xL was positive in 42 cases (88%) (Figure 4, E and F), the intensity of the expression varied from case to case (++ to ++++). Expression of p21 in >10% of the tumor cells was observed in 18 cases

(38%). The percentage of p21+ cells ranged from 14 to 100% with a median of 39%. The proliferation rate varied from <5 to 80% with a median value of 15% as assessed by MiB1 staining.

Figure 3. Immunohistochemical analysis of cyclin D1 in primary MM cases. **A:** The majority of tumor cells show strong nuclear positivity for cyclin D1 (+++) (case 29, group 2). **B:** MM with lymphoplasmacytoid features (case 32, group 2). The majority of tumor cells show strong nuclear positivity for cyclin D1 (+++). **C:** MM case with nuclear positivity in 20 to 50% of tumor cells (++) and co-expression of Stat 3P (case 20, group 1). **D:** MM with nuclear positivity in 20 to 50% of tumor cells (++) (case 35, group 2). Note that the intensity of the staining varies from cell to cell. **E:** MM with nuclear positivity in 10 to 20% of tumor cells (+) and expression of Stat 3P (case 19, group 1). **F:** MM negative for cyclin D1. Note the presence of rare, positive plasma cells (arrows) (case 46, group 3). Immunoperoxidase; original magnifications: $\times 200$ (**B**); $\times 400$ (**A**, **C-F**).

Quantitation of Stat3 and Cyclin D1 mRNA in MM Cases

Stat 3 and cyclin D1 mRNA expression levels quantitated by real-time TaqMan RT-PCR analysis are summarized in

Table 3. Under the selected experimental conditions, reproducible quantitative RT-PCR results were obtained in almost all cases. The observed Stat 3/TBP mRNA ratios were generally low (median, 0.94; range, 0.15 to 2.8), when compared to the additionally investigated control

Figure 4. Immunohistochemical analysis of anti-apoptotic proteins in MM cases. **A:** Mcl-1 expression in a MM case with activated Stat 3 (case 17, group 1). Note the strong cytoplasmic staining. **B:** Mcl-1 expression in a MM case with cyclin D1 overexpression. Note the weaker cytoplasmic positivity in comparison with the previous case and with the small reactive lymphocytes (arrows) (case 34, group 2). **C** and **D:** Bcl-2 expression in MM cases. **C:** Bcl-2 is strongly expressed in the cytoplasm in the vast majority of tumor cells. **D:** An example of a MM case negative for Bcl-2. Note the Bcl-2 positivity of a reactive lymphocyte used as internal control. **E** and **F:** Bcl-xL expression in MM cases. **E:** Bcl-xL is positive in the cytoplasm of the tumor cells. **F:** MM with lack of expression of Bcl-xL. Note the positivity in the cytoplasm of reactive histiocytes used as internal control. Immunoperoxidase; original magnifications, $\times 400$.

lymph node, spleen, and bone marrow samples (mean, 1.27; range, 1.15 to 1.38). In contrast to Stat3, mRNA cyclin D1 transcript levels showed remarkable variation in the tumors. The mRNA levels clustered in three different groups: 1) high cyclin D1 transcript levels with cyclin

D1/TBP ratios greater than 100 were observed in 11 of 48 (23%) cases, (range, 101 to 425; median, 163); 2) intermediate cyclin D1 transcript levels with cyclin D1/TBP ratios between 10 to 20 were observed in 6 of 48 (12.5%) cases (range, 10 to 18.72; median, 15.3); and 3) negative

or low cyclin D1 transcript levels with cyclin D1/TBP ratios less than 7.0 were found in 31 of 48 (64.5%) cases. The correlation between immunohistochemistry and real-time RT-PCR was excellent (Table 3). The 11 cases with strong (+++) nuclear cyclin D1 staining in the majority of the tumor cells, expressed high levels of cyclin D1 mRNA (cyclin D1/TBP ratio greater than 100). In contrast, cases with 20 to 50% cyclin D1-positive cells showed intermediate cyclin D1 mRNA expression (cyclin D1/TBP ratio between 10 to 20).

Correlation between Stat 3P and Cyclin D1 Expression

Three different groups determined by the expression of Stat 3P and cyclin D1 (protein and mRNA) were identified. In general, cases expressing cyclin D1 did not express Stat 3P and vice versa. This negative association between cyclin D1 and Stat 3P was statistically significant ($P = 0.0066$).

Group 1 contained 23 (48%) cases that revealed activated expression of Stat 3 in the majority of tumor cells; however, none of the cases showed increased expression of Stat 3 mRNA (Table 3). This finding indicates that the increased levels of activated Stat 3 in MM cases is most probably a consequence of posttranscriptional changes. Three cases in this group showed intermediate to low cyclin D1 protein and/or mRNA expression. Group 2 included the 15 (31%) cases with cyclin D1 staining and absence of activated Stat 3. Group 3 included 10 (21%) cases with neither expression of cyclin D1 nor activated Stat 3.

Correlation between Activated Stat3 and Cyclin D1 Expression with Clinicopathological Characteristics and Outcome

The tumor morphology and the clinicopathological characteristics of the patients were analyzed in the three different groups described above (Tables 2 and 3). Group 1 included 23 cases of MM with Stat 3 activation. Eleven patients were male and 12 were female with a median age of 64 years (range, 32 to 79 years). Histologically, 17 cases were composed of well-differentiated plasma cells and 6 cases of intermediately differentiated plasma cells. Three of the cases (13%) showed lymphoplasmacytoid differentiation, including case 18, which expressed cyclin D1. Twelve of 20 (60%) patients presented with stage IIIA-B disease. Twelve patients are alive 4 to 96 months later (mean, 26 months), two with no evidence of disease. Six patients died with disease and five cases were lost to follow-up. Group 2 included 15 cases with cyclin D1 expression and no activation of Stat 3 protein. However, in eight cases cytoplasmic expression of steady-state Stat 3 was observed. Ten patients were male and five were female with a median age of 64 years (range, 40 to 81 years). Histologically, all cases were composed of well-differentiated plasma cells; seven cases (47%) revealed lymphoplasmacytic differentiation.

Table 4. Proliferation Rate and Expression of Anti-Apoptotic Proteins in MM According to Groups

Antibody	Group 1 Stat 3P+	Group 2 CycD1+	Group 3 Stat 3P/ CycD1–
MiB1 M (range)	18% (5–80)	15% (5–70)	20% (5–70)
Bcl-2 (%pos)	61%	73%	70%
Bcl-xl (%pos)	90%	73%	100%
Mcl-1 (%pos)	100%	100%	100%
p21 (>10%) (%pos)	39%	47%	20%

M, median; %pos, percentage of positive cases.

Eight of 13 (62%) patients presented with stage IIIA-B disease. Nine patients are alive with disease 1 to 91 months later (mean, 36 months). Four patients died with disease and two were lost to follow-up. Group 3 included 10 cases with no cyclin D1 expression or Stat 3 activation. Five of these cases showed cytoplasmic expression of Stat 3. Four patients were male and six female with a median of 66 years (range, 47 to 76 years). Histologically, seven cases were composed of well-differentiated plasma cells and three cases of intermediately differentiated plasma cells. Eight of nine (89%) patients presented with stage IIIA-B disease. Four patients are alive with disease 27 to 54 months later (mean, 37.5 years). Two patients are dead and four patients were lost to follow-up.

No significant differences in the frequency of the main clinical parameters, ie, stage III (60% versus 62% versus 89%) and overall survival (67% versus 69% versus 67%) were observed between groups 1, 2, and 3. Nevertheless, it is of clinical interest that MM cases with cyclin D1 expression showed a male predominance (male:female, 2:1). In addition, cyclin D1+ cases were always well differentiated, and were more likely to show lymphoplasmacytoid differentiation (chi-square = 9.55).

Correlation between Activated Stat3 and Cyclin D1 Expression and Proliferation Rate and Expression of Anti-Apoptotic Proteins

The proliferation rate and the expression of anti-apoptotic proteins were analyzed separately in the three different groups described above (Table 4). There were no significant differences among the three groups concerning proliferation rate, which showed a wide range in all groups, and expression of anti-apoptotic proteins. However, Bcl-2-positive cases showed a lower proliferation rate (mean, 10%) than cases that were Bcl-2-negative (mean, 30%), and this difference was statistically significant ($P = 0.0003$) (Figure 5). The best discriminatory cutoff for proliferation rate was 20%. Cases with proliferation rate greater than 20% usually lacked expression of Bcl-2 and this was significant ($P = 0.0001$). Although there were no significant differences between the three

Figure 5. Comparison of proliferation rate in Bcl-2-positive and Bcl-2-negative cases. Statistical analysis was done using the Mann-Whitney *U*-test. Cases that are Bcl-2-positive have a mean proliferation rate of 10%, whereas cases with Bcl-2-negative have a mean proliferation rate of 30% ($P = 0.0003$).

groups and expression of anti-apoptotic proteins, cases expressing cyclin D1, tended to have a lower proliferation rate, to express more often Bcl-2 and p21, and less frequently Bcl-xL.

Discussion

In this study we analyzed in a series of MM cases the frequency of constitutively activated Stat 3 and the downstream effects of activated Stat 3 on proliferation and induced apoptosis. In addition, we explored the relationship of cyclin D1 expression to Stat 3 activation and assessed their possible influence in the pathogenesis of MM. We found activated Stat 3 in almost half (48%) of the investigated MM cases, but its presence had no influence on the proliferation rate or the expression levels of anti-apoptotic proteins. In addition, we showed that cyclin D1 overexpression and Stat 3 activation usually are mutually exclusive events in the pathogenesis of MM ($P = 0.0066$). No significant differences in the frequency of the main clinicopathological parameters analyzed were found among cases expressing Stat 3P, cyclin D1, or none of them.

Our immunohistochemistry data are in agreement with a recent study by Catlett-Falcone and colleagues,⁷ who also found a dramatic elevation of activated Stat 3 in approximately one-third of their MM cases, using an electrophoretic mobility shift assay for the detection of Stat 3 DNA-binding activity. However, there are some differences between the two studies. First, in addition to the expression of Stat 3P, we investigated the Stat 3 mRNA expression levels and found that none of the cases had elevated mRNA expression when compared to normal bone marrow and lymphoid tissue, indicating that the strong protein expression of Stat 3 is a consequence of posttranscriptional changes. Second, they demonstrated that activated Stat 3 induces Bcl-xL expression and confers resistance to Fas-induced apoptosis in the U266 MM cell line, and proposed that the Stat 3 pathway may be a main source of anti-apoptotic signaling in MM. Although we also identified expression of anti-apoptotic proteins (ie, Bcl-xL, Bcl-2, and Mcl-1), in most cases, the expres-

sion of these proteins was independent of the expression and activation of Stat 3. This is an unexpected finding in the light of previous *in vitro* observations that have shown that the expression of Bcl-xL and Mcl-1 are tightly regulated by IL-6, most probably in a Stat 3-dependent mechanism.^{7,8,10} However, most of the data concerning the role of IL-6/gp130/Stat 3 pathway for the survival and proliferation of human MM cells has been obtained through experiments performed with cell lines. It is interesting to note that our study is not the first study that has challenged the role of the IL-6/gp130/Stat 3 pathway in the pathogenesis of MM.^{9,11,21,22} Ogata and colleagues²¹ found that both Stat 1 and Stat 3 seem to be constitutively active in IL-6 responsive and nonresponsive MM cells, and stimulation of cells with IL-6 does not increase the activity of Stat 3. Our results are in agreement with these latter findings in that in MM cell lines, the presence of autocrine IL-6 does not seem to induce the phosphorylation or activation of Stat 3, because we identified Stat 3P only in two of the five IL-6-positive cell lines, including the well-described U266 line.^{11,21} Furthermore, a recent study has shown that the Stat 3 pathway is not essential for the survival of human MM cells.²² The authors demonstrated that MM cells become independent of the IL-6/gp130/Stat3 pathway in the presence of bone marrow stromal cells. Accordingly, all cell lines showed with some variation, high levels of Bcl-xL and Mcl-1, regardless of the presence of IL-6 or phosphorylated Stat 3, paralleling our results in the primary MM cases. Taken together, our data suggest that the activation of Stat 3 does not seem to be the key player in the anti-apoptotic and survival signaling in MM.

The high expression of Mcl-1 in all cell lines and primary MM cases found in this study, regardless of the expression of activated Stat 3, suggests that its regulation has become Stat 3-independent, and raises important questions concerning the role of Mcl-1 for the pathogenesis of MM. Accordingly, in a recent study, preliminary data showed that freshly isolated CD138+ MM cells express high levels of Mcl-1 protein, which are maintained for up to 24 hours, independent of IL-6.⁹ In addition, the presence and function of Mcl-1 seems to be of central importance for the survival of MM cells, because rapid down-regulation of Mcl-1 protein levels by anti-sense oligonucleotides leads to apoptosis of MM cells. In contrast, the down-regulation of Bcl-2 and Bcl-xL does not affect the viability of myeloma cells.^{9,23} Critical issues remain to be resolved, including the extent, if any, to which activated Stat 3 regulates anti-apoptotic proteins in MM, and whether or not the universal presence of Mcl-1 in MM cells is because of constitutive expression, as suggested by our results and that of others.^{9,10,23}

Bcl-2, another anti-apoptotic member of the Bcl-2 family, is also highly expressed in malignant plasma cells.^{24,25} In contrast to Bcl-xL and Mcl-1, the expression of Bcl-2 showed a close correlation with the proliferation rate. Highly proliferative cases tended to be Bcl-2-negative ($P = 0.0003$). Our findings, in both cell lines and primary cases, support previous observations suggesting that the activation of Bcl-2 is independent of the IL-6-Jak/Stat pathway.^{9,23,24} Although, Bcl-xL, Mcl-1, and

Bcl-2 have anti-apoptotic functions, it seems that each of them has unique roles, and most probably complementary activities in cell death regulation in MM. This is in line with their differential expression during various stages of cell differentiation in lymphoid tissues.^{26,27}

Another important finding of this work is that cyclin D1 overexpression and Stat 3 activation are in general, mutually exclusive events in MM. In some experimental systems using rodent fibroblast cell lines, a constitutively active Stat 3 construct was capable of up-regulating cyclin D1 expression at the level of transcription, increasing the mRNA levels up to fivefold.^{2,6} However, the consequences of activated Stat 3 have not been investigated in lymphoid cells where cyclin D1 is not normally expressed. This prompted us to investigate whether constitutively active Stat 3 could activate cyclin D1 in MM, and eventually explain the dysregulation of cyclin D1 in those cases in which no molecular alterations of the bcl-1 locus are found. However, in 20 of 23 cases with constitutive activation of Stat 3, no elevation of cyclin D1 mRNA was observed, indicating that at least in MM, Stat 3 is not capable of activating cyclin D1. Interestingly, all cases expressing high levels of cyclin D1 mRNA were negative for Stat 3P. The reason(s) for the lack of Stat 3P in cases expressing cyclin D1 are uncertain, but there are at least two possible explanations. One explanation is that in MM, the high levels of cyclin D1 could make the activation of Stat 3 redundant. A second, and more interesting possibility is that the high levels of cyclin D1 could lead to the down-regulation of Stat 3 as part of a negative feedback mechanism of the Jak/Stat pathway. This possibility is supported by a recent study showing that overexpressed cyclin D1 interacts with Stat 3 protein and inhibits its transcriptional activity.²⁸

Our findings in MM, in which high levels of Stat 3P are associated with lack of expression of cyclin D1 at the protein and mRNA level, contrast with recent observations in head and neck squamous cell carcinoma, in which constitutive activation of Stat 3 correlates with overexpression of cyclin D1.²⁹ Furthermore, the authors suggest that activation of Stat 3 plays a causative role in the overexpression of cyclin D1. The reason for this difference is not clear; however, it may reflect tissue-specific differences in the cell cycle machinery. In contrast to epithelial cells, plasma cells do not normally express cyclin D1, and the molecular consequences of aberrant expression of cyclin D1 in these cells may be different. We have observed a similar phenomenon in mantle cell lymphoma in which the dysregulation of cyclin D1 is accompanied by loss of expression of the cyclin-dependent kinase inhibitor p27^{Kip1},³⁰ in contrast to breast carcinoma in which overexpression of cyclin D1 correlates with high expression of p27^{Kip1}.³¹

In conclusion, constitutive activation of Stat 3 is present in a significant proportion of primary MM cases. However, this does not seem to have a major impact on the expression of anti-apoptotic proteins and proliferation. The universal expression of Mcl-1 in MM cell lines and primary cases, irrespective of Stat 3P, indicates that its expression is constitutive, calling into question the role of Stat 3 for its activation. An intriguing finding is the mutual exclusion of Stat 3P and cyclin D1 overexpres-

sion, hinting to a so far unexplored function of cyclin D1 as negative regulator of the Jak/Stat pathway.

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