

Expression of eight genes of nuclear factor-kappa B pathway in multiple myeloma using bone marrow aspirates obtained at diagnosis

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Summary. Purpose: To evaluate the expression of NF- κ B pathway genes in total bone marrow samples obtained from MM at diagnosis using real-time quantitative PCR and to evaluate its possible correlation with disease clinical features and survival. Material and methods: Expression of eight genes related to NF- κ B pathway (*NFKB1*, *IKB*, *RANK*, *RANKL*, *OPG*, *IL6*, *VCAM1* and *ICAM1*) were studied in 53 bone marrow samples from newly diagnosed MM patients and in seven normal controls, using the Taqman system. Genes were considered overexpressed when tumor expression level was at least four times higher than that observed in normal samples. Results: The percentages of overexpression of the eight genes were: *NFKB1* 0%, *IKB* 22.6%, *RANK* 15.1%, *RANKL* 31.3%, *OPG* 7.5%, *IL6* 39.6%, *VCAM1* 10% and *ICAM1* 26%. We found association between *IL6* expression level and International Staging System (ISS) ($p=0.01$), meaning that MM patients with high ISS scores have more chance of overexpression of *IL6*. The mean value of *ICAM1* relative expression was also associated with the ISS score ($p=0.02$). Regarding OS, cases with *IL6* overexpression present worse evolution than cases with *IL6* normal expression ($p=0.04$). Conclusion: We demonstrated that total bone marrow aspirates can be used as a source of material for gene expression studies in MM. In this context, we confirmed that *IL6* overexpression was significantly associated with worse survival and we described that it is associated with high ISS scores. Also, *ICAM1* was overexpressed in 26% of cases and its level was associated with ISS scores.

Key words: Multiple myeloma, International staging System, Nuclear factor-kappa B, Interleukin-6, Prognosis

Introduction

Multiple myeloma (MM) is a B-cell malignancy characterized by expansion of monoclonal plasma cells in bone marrow and is associated with osteolytic bone disease (Berenson et al., 2001). Currently, MM remains as an incurable disease despite treatment with conventional and/or high-dose chemotherapy (Kyle and Rajkumar, 2004). Therefore, novel therapies that target both tumor cells and bone marrow microenvironment are urgently needed to overcome resistance to conventional therapies and improve MM patient's outcome (Chauhan et al., 2005a).

Members of the nuclear factor-kappa B (NF- κ B) transcription factor family play an important role in the pathogenesis of lymphoid malignancies (Berenson et al., 2001) and also have a role in chemoresistance of a variety of tumors (Bharti et al., 2004). In MM, NF- κ B is a key regulator, promoting expansion of tumor cells in the bone marrow milieu and is involved in the control of various cellular processes, such as immune and inflammatory responses, cell growth, bone resorption, and apoptosis. NF- κ B is bound to I- κ B in the cytoplasm and its activation occurs through proteasome-mediated degradation of I- κ B (Mitsiades et al., 2002; Rajkumar et al., 2005). This allows NF- κ B to translocate to the nucleus and bind to specific cis-acting consensus sequences (κ B sites) located in the regulatory region of inducible genes, including cytokines (TNF α , IL-2, IL-6, IL-8, α -interferon), adhesion molecules (intercellular adhesion molecule-1 or ICAM-1, vascular cell adhesion

molecule-1 or VCAM-1), genes involved in the regulation of apoptosis (*TRAF1/2*, *cIAP1* and *2*, *TNRF1*, *I κ B*) or response to NF- κ B and those involved in cell cycle regulation and proliferation (*cyclin D1*, *MYC*, *p53*, *Rb*) (Willis and Dyer, 2000). Also, adhesion of MM cells to bone marrow stromal cells triggers NF- κ B-mediated transcription and secretion of interleukin-6 (IL-6), which has an important role in enhancing growth or prolonging survival of MM cells (Bharti et al., 2004; Roodman, 2004).

Since MM cells have altered or defective cell cycle proteins, leading to an increased proliferation rate and increased accumulation of damaged proteins, they have higher dependency on the proteasomal degradation process. Therefore, proteasome inhibitors, such as bortezomib, down-regulate NF- κ B activation and related cytokine production, thereby enhancing the cytotoxic effects of bortezomib-based chemotherapy (Chauhan et al., 2005b).

Genes important to MM osteolytic lesions pathogenesis also participate in the NF- κ B pathway (Giuliani et al., 2006). The bone marrow micro-environment plays a critical role in the formation of osteoclasts, through production of essential cytokines for osteoclasts biology (Roodman, 2004). Osteoclast precursors express RANK, a member of the tumor necrosis factor receptor superfamily. RANKL binds the RANK receptor and induces the differentiation of osteoclasts through the NF- κ B and Jun N-terminal kinase (JNK) pathways (Roodman, 2004). Also, in the bone marrow microenvironment, osteoblasts secrete osteoprotegerin (OPG), which acts as a decoy receptor and blocks RANKL, preventing RANK activation (Sezer et al., 2003).

The outcome of patients with MM is highly variable because there is a heterogeneity in both myeloma cell biology and host factors (Berenson et al., 2001). A new staging system, based on serum levels of β 2-microglobulin and serum albumin at diagnosis, was established as the International Staging System (ISS) and seems to reflect the genetic-molecular background in MM (Greipp et al., 2005). Therefore, the aim of this study was to evaluate the expression of NF- κ B pathway genes in MM patients by real-time quantitative PCR and its possible correlation with clinical features, including ISS and survival.

Materials and methods

Patients and controls

Between June 2002 and April 2006, we obtained total bone marrow aspirates from 53 newly diagnosed MM patients, referred to the Department of Hematology and Hemotherapy of Federal University of São Paulo, UNIFESP, São Paulo, Brazil. Patients were included after diagnosis of MM based on The International Working Group Criteria (The International Myeloma Working Group, 2003) and after signature of informed

consent. Bone marrow biopsy and aspiration were obtained from the posterior iliac crest. Only patients with no previous chemotherapy, corticosteroids or bisphosphonates were included. MM patients were classified according to Durie-Salmon staging system and ISS (Greipp et al., 2005). Seven healthy controls were used, being six bone marrow aspirates from allogeneic transplant donors and one pool of 10 bone marrow samples from Clontech (Palo Alto, CA, USA). Written informed consent was obtained from all patients and controls, and the study was approved by our Institution Ethical Committee. Clinical characteristics of the MM patients are shown in Table 1.

Pilot study

Tumor plasma cells were isolated from 12 total bone marrow aspirates obtained from MM cases at diagnosis through magnetic sorting of CD-138 (syndecan-1) positive cells using MACS system (Magnetic Cell Sorting of Human Cells, Miltenyi Biotec, Bergisch Gladbach, Germany). Normal plasma cells were

Table 1. Clinical characteristics of 53 MM patients included at diagnosis.

Clinical characteristics	N	%
Age: median (range)-62 years (27-80)		
<62 years	25	47
≥ 62 years	28	53
Sex		
Male	31	58.5
Female	22	41.5
M-component type		
IgG kappa or lambda	30	56.6
IgA kappa or lambda	13	24.5
Light chain only	10	18.8
Durie Salmon stage		
IA	2	3.8
IIA	2	3.8
IIIA	32	60.4
IIIB	17	32.1
ISS		
1	10	18.9
2	18	34
3	21	39.6
Missing data	4	7.5
Bone marrow plasma cells (median:80%)		
<50%	8	15.1
50-79%	13	24.5
≥ 80%	32	60.4
Treatment		
Conventional chemotherapy*	46	86.8
High dose melphalan + ASCT**	5	9.4
Allogeneic transplant	2	3.8
Total	53	100

*Conventional chemotherapy: VAD or Melphalan+Prednisone or Thalidomide+dexamethasone. **High dose melphalan and autologous stem cell transplantation (ASCT) after induction treatment.

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obtained from three pools of palatine tonsils (children submitted to tonsillectomy) and two bone marrow aspirates from allogeneic transplant donors through magnetic sorting of CD-138 (syndecan-1) positive cells. Ten out of 12 MM bone marrow aspirates had more than 50% of plasma cells. The magnetic sorting method was able to separate tumor plasma cells from other bone marrow cells achieving a high degree of purification (>85%), confirmed by flow cytometry.

Genes selected for study

We chose eight genes related to the NF- κ B pathway for this study. They are related to cellular proliferation (*NFKB1*, *NFKBIA* (*IKB*), *IL6*), bone disease (*RANK*, *RANKL*, *OPG*) and interaction between plasma cells and stromal cells (*VCAM1*, *ICAM1*) (Table 2).

RNA extraction and cDNA synthesis

Bone marrow aspirates were obtained in EDTA in the same place where bone marrow biopsies were performed. Two-hundred μ L of total bone marrow aspirates were submitted to erythrocytes lysis. The pellet was homogenized in 1mL of TRIzol reagent (Invitrogen, Carlsbad, CA) and maintained at -70°C until total RNA was extracted. The RNA was recovered from the aqueous phase by ethanol precipitation and the pellets were dissolved in 30 μ L of DEPC water. First-strand cDNA synthesis, primed with oligo(dT) and 2 μ g of RNA template, was reverse transcribed (Superscript II, Invitrogen, Carlsbad, CA, USA). After cDNA synthesis, the expression of *Notch2* was determined in all samples and the quality of samples was assessed by 2% agarose gel electrophoresis.

Real-time quantitative PCR

Gene expression was evaluated by real-time quantitative PCR (RQ-PCR) on ABI PRISM[®] 7000 Sequence Detection System Instrument (Applied Biosystems, Foster City, CA, USA). Primers and probes were obtained through Assays-by-Design Service (Applied Biosystems). Each cDNA sample was normalized using glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene based on previous publications (Condomines et al., 2007; Gonzalez-Paz et

al., 2007). TaqMan Universal PCR Master Mix (Applied Biosystems) was used to perform RQ-PCR and assays were reproduced in triplicate. Blank and positive controls were run in parallel to verify amplification efficiency. Genes were considered differentially expressed in tumor samples when their expression levels showed at least a 4-fold increment or decrease in comparison to average expression in normal samples.

Statistical analysis

Associations between the studied variables were tested by the Pearson Chi-Square Test (χ^2). T-Test or ANOVA were used to perform mean comparisons. Overall survival (OS) time was calculated from the date from diagnosis of MM until death or last follow-up. Survival curves of the eight studied genes were obtained separately according to their expression level (overexpression, normal expression and under-expression). Actuarial probabilities of OS were estimated according to the Kaplan-Meier method and the curves were compared using the log-rank test. The level of significance for all statistical tests was 5%.

Results

Our initial analyses were performed in a restricted group of 12 MM patients to compare the expression of the eight selected genes in total bone marrow aspirates and their respective purified tumor plasma cells. Median tumor plasma cell infiltration in this small group of bone marrow aspirates was 47% (ranging from 10 to 80%). Also, ten out of 12 cases had more than 50% of plasma cells infiltration assessed by bone marrow biopsy performed in the same place of aspirate. We found underexpression of *RANKL*, *OPG* and *VCAM1* in 100%, 83.3% and 92% of purified tumor plasma cells, respectively. On the other hand, expression of *IL6* showed 58.3% of concordance between the two sources of RNA (total bone marrow aspirate and purified tumor plasma cells) ($r=0.838$) (Table 3). This pilot study showed that *IL6* overexpression was detected in both sources of samples, and genes related to bone marrow microenvironment were underexpressed (*OPG*, *RANKL*, *VCAM1*) when only purified plasma cells were evaluated. Therefore, we decided to study these eight NF- κ B pathway genes using total bone marrow aspirates

Table 2. Nuclear factor-kappa B pathway studied genes in MM patients.

<i>NFKB1</i>	Nuclear factor-kappa B (p105)	NM_003998	Transcription regulator of several biological functions
<i>IKB</i> (<i>NFKBIA</i>)	Nuclear factor-kappa B Inhibitor	NM_020529	Inhibits NF- κ B in cytoplasm
<i>RANK</i>	NF κ B activator	NM_003839	Osteoclast differentiation
<i>RANKL</i>	RANK ligand	NM_003701	Osteoclast activation and differentiation
<i>OPG</i>	Osteoprotegerin	NM_002546	Negative regulator of bone resorption
<i>IL6</i>	Interleukin-6	NM_000600	MM cells proliferation
<i>VCAM1</i>	Vascular cell adhesion molecule	NM_001078	Cellular adhesion and sign transduction
<i>ICAM1</i>	Intercellular adhesion molecule	NM_000201	Cellular adhesion

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Table 3. Expression of NF-κB genes in 12 total bone marrow samples and respective purified MM plasma cells. Genes were considered differentially expressed in tumor samples when their expression levels showed at least a 4-fold increment or decrease in comparison to normal samples.

Case	%BM PC		RANK		RANKL		OPG		NFKB1		IKB		IL6		VCAM1		ICAM1	
	Aspirate	Biopsy	BM	PC														
17	70	90	Gray	White	Gray	White	Gray	White	Gray	White	Gray	Gray	White	White	White	White	White	White
20	28	98	Gray	Gray	Black	Black	Gray	White	Gray	White	Gray	Black	Black	Black	Gray	White	White	White
22	20	95	Black	Gray	Gray	White	Black	Black	White	White	White	White						
23	60	95	Gray	White	White	White	Black	Gray	White	White	White	White	White	White	Black	White	White	White
24	10	10	White	Black	Black	White	White	White										
27	52	80	White															
28	50	70	White	White	White	White	Gray	White	White	White	White	Black	White	Black	White	White	Black	White
32	33	50	White															
34	10	30	White	White	Black	Black	White	Gray	White									
35	53	70	White															
44	80	90	Black	Black	Black	Black	White	White	White	White	White	Black	Black	Black	White	White	White	White
46	43	70	Gray	White	Black	White	White	White	White									

BM: bone marrow samples; PC: plasma cells; White: Underexpression; Gray: Normal expression; Black: Overexpression.

Table 4. Correlation between gene expression level and ISS in total bone marrow aspirates of MM patients.

Gene expression		ISS 1	ISS 2	ISS 3	p value
RANK	Underexpression	1(10)	0(0)	0(0)	0.181
	Normal expression	9(90)	14(77.8)	18(85.7)	
	Overexpression	0(0)	4(22.2)	3(14.4)	
	Total	10(100)	18(100)	21(100)	
RANKL	Underexpression	1(14.3)	1(5.9)	1(5)	0.817
	Normal expression	5(71.4)	11(64.7)	12(60)	
	Overexpression	1(14.3)	5(29.4)	7(35)	
	Total	7(100)	17(100)	20(100)	
OPG	Underexpression	1(10)	4(22.2)	8(38.1)	0.485
	Normal expression	8(80)	13(72.2)	11(52.4)	
	Overexpression	1(10)	1(5.6)	2(9.5)	
	Total	10(100)	18(100)	21(100)	
NFKB1	Underexpression	0(0)	0(0)	0(0)	(a)
	Normal expression	10(100)	18(100)	21(100)	
	Overexpression	0(0)	0(0)	0(0)	
	Total	10(100)	18(100)	21(100)	
IKB	Underexpression	0(0)	0(0)	0(0)	0.162
	Normal expression	10(100)	13(72.2)	15(71.4)	
	Overexpression	0(0)	5(27.8)	6(28.6)	
	Total	10(100)	18(100)	21(100)	
IL6	Underexpression	0(0)	0(0)	4(19)	0.010
	Normal expression	9(90)	7(38.9)	9(42.9)	
	Overexpression	1(10)	11(61.1)	8(38.1)	
	Total	10(100)	18(100)	21(100)	
IL6	Under and normal expression	9(90)	7(38.9)	13(61.9)	0.029
	Overexpression	1(10)	11(61.1)	8(38.1)	
	Total	10(100)	18(100)	21(100)	
VCAM1	Underexpression	1(11.1)	1(5.6)	4(21)	0.483
	Normal expression	7(77.8)	15(83.3)	13(68.4)	
	Overexpression	1(11.1)	2(11.1)	2(10.6)	
	Total	9(100)	18(100)	19(100)	
ICAM1	Underexpression	0(0)	0(0)	0(0)	0.112
	Normal expression	9(100)	14(77.8)	11(57.9)	
	Overexpression	0(0)	4(22.2)	8(42.1)	
	Total	9(100)	18(100)	19(100)	

(a) No statistics are computed because *NFKB1* is a constant.

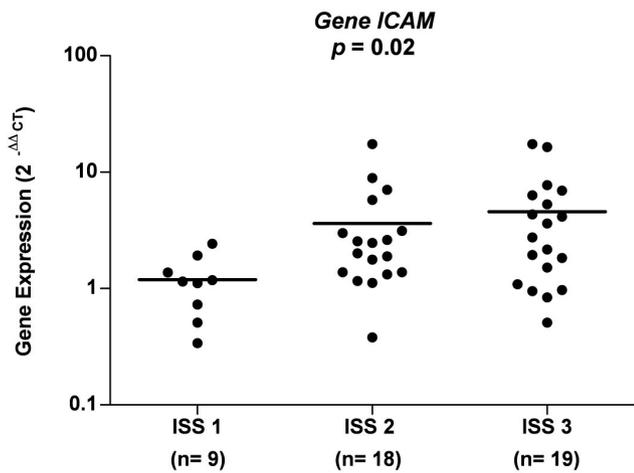
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Fig. 1. Relative expression of gene *ICAM1* according to ISS 1, 2 and 3 in total bone marrow aspirates of MM patients.

to collect simultaneous information regarding microenvironment and tumor cells status.

We studied the expression of the eight selected genes in 53 total bone marrow samples and in seven normal samples. Percentages of overexpression of the eight studied genes were: *NFKB1* 0%, *IKB* 22.6%, *RANK* 15.1%, *RANKL* 31.3%, *OPG* 7.5%, *IL6* 39.6%, *VCAM1* 10% and *ICAM1* 26%. We correlated each gene expression level (overexpression, normal expression and underexpression) to bone marrow plasma cells percentage and to ISS system (Table 4). In 53 studied patients, only eight had less than 50% of plasma cells in bone marrow biopsy and we did not find a correlation between gene expression level and the number of plasma cells at diagnosis. However, we found an association between *IL6* expression level and ISS ($p=0.01$), meaning that patients with advanced stage disease have more chance of presenting *IL6* overexpression.

Mean values of gene expression were compared in the three ISS categories. The mean value of *ICAM1* relative expression was associated with ISS score ($p=0.02$), i.e., cases classified as ISS 3 had higher levels of *ICAM1* expression (Fig. 1).

In this study, follow-up varied from 4 days to 60 months (median=20 months). Median overall survival (OS) for the entire group was 21 months. We did not find any statistical difference between survival curves of clinical parameters. We found a statistical difference between *IL6* expression level and survival, i. e., cases with *IL6* overexpression presented worse evolution than cases with *IL6* normal expression ($p=0.04$). Surprisingly, cases with underexpression of *IL6* had shorter survival than cases with normal expression of *IL6* ($p=0.003$). These results were confirmed after exclusion of patients submitted to autologous stem cell transplantation (Fig. 2). Besides *IL6*, we analyzed survival curves comparing *IKB* ($p=0.83$), *RANK* ($p=0.44$), *RANKL* ($p=0.07$), *OPG*

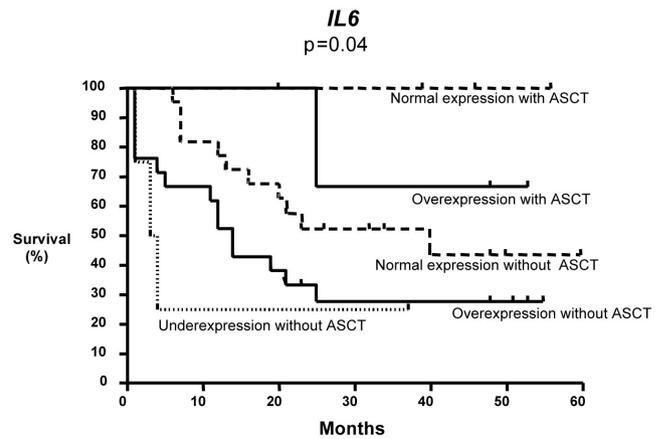


Fig. 2. Survival curves according to *IL6* expression level at diagnosis and autologous stem cell transplant (ASCT) in 53 MM patients ($p=0.04$). There was no underexpression case with ASCT.

($p=0.31$), *VCAM1* ($p=0.27$) *ICAM1* ($p=0.95$) expression level and we did not find any statistical difference among survival curves.

Discussion

Extensive studies have demonstrated the importance of the bone marrow microenvironment in promoting cell growth, survival and drug resistance in MM. They have already proposed several therapies based on targeting the MM cells and their interactions in bone marrow milieu (Richardson et al., 2007). To be effective, ideal therapies must include the molecular mechanisms underlying the protective effects of the bone marrow microenvironment on MM cells (Richardson et al., 2007). Thus, due to the importance of bone marrow microenvironment to MM pathogenesis we conducted a pilot study to assess which would be the best source of RNA for these eight NF- κ B pathway gene expression studies. In this small group of patients we did not find concordance between NF- κ B pathway genes expression in bone marrow aspirates and isolated plasma cells, except *IL6*. We concluded that isolated analyses of purified MM plasma cells would miss information regarding the studied genes expressed predominantly by other bone marrow cells, such as stromal cells. Of course it was possible because we studied a selected number of genes and it cannot be extrapolated to more complex global gene expression analysis methods, such as SAGE or microarrays. Also, the majority of patients included in this study had more than 50% of plasma cells infiltration in bone marrow. This characteristic probably reduced the risk of underexpression of a given gene due to low percentage of plasma cell infiltration. Therefore, because of underexpression of *RANKL*, *OPG* and *VCAM1* in isolated plasma cells, we decided to study total bone marrow aspirates. In the literature, there is no consensus

about the cellular origin (stromal/osteoblastic or MM cells) of RANKL increased biological activity in MM (Haaber et al., 2007). Another possible strategy to be adopted in future in this kind of study would be to examine the expression of the same genes in two populations, separately: CD 138-positive population (obtained by magnetic sorting) and CD 138-negative population (flow through).

According to the Durie & Salmon staging system, 92.5% of patients were classified as stage III, reflecting a possible delay in suspecting MM before patients are referred to our institution - a tertiary service in an underdeveloped country. Unfortunately, in this cohort of consecutive patients, we did not have samples of monoclonal gammopathy or early stage MM to compare the results obtained with MM stage III. However, we were able to classify our patients better through ISS staging system: 34% were ISS 2 and 39.6% ISS 3. After 60 months of maximum follow-up, median overall survival was 21 months for our entire group (31.5 months for ISS 1, not reached for ISS 2 and 13 months for ISS 3).

IL6 was overexpressed in 39.6% of our patients and it was significantly associated to advanced-stage disease. IL-6 was originally characterized as a cytokine that causes the terminal differentiation of activated B-cells into immunoglobulin-secreting cells and was then shown to be a major growth and survival factor for MM cells. More recently, IL-6 has been shown to stimulate the growth of plasmablasts and to protect early plasma cells from apoptosis, allowing their terminal differentiation into plasma cells (Brocke-Heidrich et al., 2004).

Besides the importance of *IL6*, the analyses of the genes of NF- κ B in MM total bone marrow samples showed overexpression of at least three more genes: *RANKL* in 31.3%, *IKB* in 22.6% and *ICAM1* in 26% of cases. Therefore, *ICAM1* seems to have some new potential as a therapeutic target in MM, since *RANKL* and *IKB* have been inhibited and stabilized by denosumab (monoclonal antibody against RANKL) (Body et al., 2006) and bortezomib, respectively (Richardson et al., 2005; Mitsiades et al., 2006). Furthermore, the mean value of *ICAM1* relative expression was associated with ISS scores, i.e., cases classified as ISS 3 had higher levels of *ICAM1*, confirming its importance in MM aggressiveness. Since this molecule stabilizes NF- κ B, we could hypothesize that overexpression of *IKB* could confer better prognosis to MM patients. Although we found overexpression of *IKB* in 22.6% of patients, this group did not correlate with ISS stage ($p=0.162$) or survival ($p=0.83$), when compared to normal or underexpression group.

We did not find any statistical difference between survival curves of clinical parameters. Probably this happened because the majority of patients presented advanced stage disease (stage III). *IL6* overexpression was related to worse survival in our study and confirms its participation in myeloma progression, regulating growth and survival of tumor cells. However, there was

no difference in survival between patients with *IL6* underexpression and overexpression. This fact probably occurred because all patients with *IL6* underexpression were ISS stage 3. Therefore, other factors related to worse prognosis must be influencing the unfavorable outcome of such MM patients, besides *IL6* expression.

Recently, Annunziata et al. (2007) described diverse genetic and epigenetic mechanisms, such as alteration of *NIK*, *TRAF3*, *CYLD*, *BIRC2/BIRC3*, *CD40*, *NFKB1*, or *NFKB2*, leading to NF- κ B activity in myeloma cell lines and patient samples. Most primary MM patient samples had evidence of NF- κ B pathway activation, suggesting that therapeutic strategies targeting the classical NF- κ B pathway should be pursued. Their work showed that targeted disruption of classical NF- κ B signaling with a small-molecule inhibitor of IKKb blocked myeloma cell proliferation and induced cell death. Their recent findings corroborate the importance of the NF- κ B pathway as a target for MM therapy, justifying all the efforts to better understand this pathway.

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