

**GENE EXPRESSION OF ANTI- AND PRO-APOPTOTIC PROTEINS IN
MALIGNANT AND NORMAL PLASMA CELLS.**

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Summary

Survival of malignant plasma cells is a key event in disease occurrence, progression and chemoresistance. Using DNA-microarrays, we analysed the expression of genes coding for 58 proteins linked with extrinsic and intrinsic apoptotic pathways, caspases and inhibitor of apoptosis proteins. We considered 6 memory B cells (MBC), 7 plasmablasts (PPC), 7 bone marrow plasma cells (BMPC) and purified myeloma cells (MMC) from 92 newly-diagnosed patients.

40 out of the 58 probe sets make it possible to separate MBC, PPC and BMPC in 3 homogeneous clusters, characterized by an elevated expression of *TNFRSF10A*, *TNFRSF10B*, *BCL2A1*, *CASP8*, *CASP9* and *PMAIP1* genes for MBC, of *FAS*, *FADD*, *AIFM1*, *BIRC5*, *CASP2*, *CASP3* and *CASP6* for PPC and of *BCL2*, *MCL1*, *BID*, *BIRC3* and *XIAP* for BMPC. Thus, B cell differentiation is associated in change of expression of pro-apoptotic and anti-apoptotic genes. Regarding MMC, the major finding is *TNFSF10* upregulation in MMC that might be counteracted by a high osteoprotegerin production by BM stromal cells and a decreased expression of *FAS*, *APAF1* and *BNIP3* compared to normal BMPC. Out of the 40 genes, *CASP2* and *BIRC5* expression in MMC had adverse prognosis in 2 independent series of previously-untreated patients.

Introduction

Multiple myeloma (MM) is a B cell neoplasia characterized by accumulation of clonal plasma cells, *i.e.* multiple myeloma cells (MMC), in the bone marrow. This accumulation depends both on survival and proliferation of MMC. Several genetic abnormalities in MM target cell cycle genes: *CCND1*, *CCND3*, *CKS1B* (Fonseca, *et al* 2004; Avet-Loiseau, *et al* 2007). Other abnormalities, t(4;14) or t(14;16), result in high cyclin D2 expression (Bergsagel, *et al* 2005). The percentage of proliferating MMC is usually low, but is nevertheless a powerful adverse prognostic factor (Greipp, *et al* 1988). Besides an increased proliferation, the survival of MMC is a prior and an essential condition to allow the accumulation of clonal plasma cells. In patients with intramedullary MM, the survival of MMC is strictly dependent upon the interaction with bone marrow (BM) stromal cells and/or osteoclasts (Uchiyama, *et al* 1993; Yaccoby, *et al* 2004). When purified *in vitro*, MMC rapidly apoptose, but addition of BM stromal cells can rescue them from apoptosis (Gu, *et al* 2000). Several growth factors are involved in this MMC/stromal cell interactions, including IL-6 (Zhang, *et al* 1992), IGF-1 (Ferlin, *et al* 2000), IL-10 (Gu, *et al* 1996), EGF family members (Mahtouk, *et al* 2004; Mahtouk, *et al* 2005), BAFF/APRIL (Moreaux, *et al* 2004), IFN- α (Ferlin-Bezombes, *et al* 1998), HGF (Derksen, *et al* 2003), Wnt family members (Derksen, *et al* 2004), IL-21 (Brenne, *et al* 2002), and Jagg/notch family members (Houde, *et al* 2004).

Two main pathways control apoptosis, the extrinsic and the intrinsic (or mitochondrial) pathways (Fig 1; see Cory *et al.* and Danial *et al.* for review) (Cory and Adams 2002; Danial and Korsmeyer 2004). The extrinsic pathway involves the death receptor family which includes receptors which possess a death domain (DD) as Fas, TRAIL-R1 (DR4), TRAIL-R2 (DR5) or TNF-R1. Extrinsic apoptosis mediated by Fas

or TRAIL has been studied in MM. Purified MMC present a variable sensitivity to Fas but unseparated MMC are Fas resistant (Westendorf, *et al* 1995) and exogenous cytokines as IL-6, being one of the main myeloma growth factors (Klein, *et al* 2003), reverse Fas-mediated apoptosis in sensitive MMC (Chauhan, *et al* 1997). MMC are highly and specifically sensitive to TRAIL-mediated apoptosis (Gazitt 1999; Mitsiades, *et al* 2001). However, it has been suggested that the expression of decoy receptors for TRAIL could block TRAIL-mediated apoptosis (Locklin, *et al* 2007). TNF- α is reported to be a growth factor for myeloma cell lines rather an apoptotic factor activating TNF-R1 receptor (Jourdan, *et al* 1999; Hideshima, *et al* 2001).

The intrinsic pathway induces a dysfunction of some organelles, in particular mitochondrial dysfunction which results in a permeabilization of their outer membrane and the concomitant release of apoptogenic proteins into the cytosol allowing the execution of the apoptotic program (Fig 1). The integrity of the outer mitochondrial membrane is controlled by the Bcl-2 family members (Kuwana and Newmeyer 2003). Today, about twenty Bcl-2 family members have been described, of which six have an anti- and 14 a pro-apoptotic activity (see list in Table I). Therefore, the cell fate is highly dependent on the balance between these two groups of proteins. Several studies have elucidated the role of anti-apoptotic proteins to trigger myeloma cell survival. Most studies confirmed the anti-apoptotic protein Mcl-1 to be essential for the survival of MMC (Puthier, *et al* 1999; Jourdan, *et al* 2000; Spets, *et al* 2002; Zhang, *et al* 2002). Our group has shown that among 10 Bcl-2 family proteins, only Mcl-1 is upregulated by IL-6 and IFN- α in several myeloma cell lines (Jourdan, *et al* 2000). In addition, a constitutive expression of Mcl-1 can prevent MMC from growth factor deprivation induced apoptosis (Jourdan, *et al* 2003). The role of two further anti-apoptotic members, Bcl-2 and Bcl-xL, remains currently unclear. Their regulation

by IL-6 is controversial and addition of antisense to Bcl-xL or Bcl-2 did not lead to myeloma cell apoptosis, unlike antisense to Mcl-1 (Derenne, *et al* 2002). Regarding the role of pro-apoptotic proteins of the Bcl-2 family, we found that IL-6, IFN- α and IGF-1 did not regulate gene or protein expression of 5 pro-apoptotic proteins (Bax, Bak, Bid, Bcl-xS and Bad) (Jourdan, *et al* 2000). Some authors have suggested that Bad phosphorylation and sequestration by 14-3-3 proteins induced by activation of the PI3K/Akt pathway by myeloma growth factors is critical to prevent myeloma cell apoptosis (Ge and Rudikoff 2000). More recently, Gomez-Bougie *et al.* have shown that the pro-apoptotic protein Bim was negatively regulated by IL-6 in MMC, and was strongly associated to Mcl-1 in viable cells (Gomez-Bougie, *et al* 2004). These authors suggest that Bim is sequestered by Mcl-1 in viable cells and that in apoptotic conditions Noxa, a “BH3 only” sensitizer proapoptotic protein, is upregulated and can displace Bim from Mcl-1, allowing Bim to activate Bak or Bax and to induce apoptosis (Gomez-Bougie, *et al* 2007).

In the present study, we have evaluated the expression of genes coding for proteins linked to extrinsic and intrinsic apoptosis, caspases and inhibitors of apoptosis proteins using Affymetrix microarrays. These expression were evaluated in purified MMC of a large panel of patients and compared with those of normal B cells, plasmablastic cells and bone marrow plasma cells.

Materials and Methods

Cell samples

MMC of 92 consecutive patients with previously-untreated MM were included in this study. This study is approved by the ethic committees at the University hospitals of Heidelberg (Germany) and Montpellier (France) and patients' written informed consent was given. These 92 patients were treated with high dose therapy (HDC) and autologous stem cell transplantation (ASCT). MMC were purified using an autoMACS with anti-CD138 MACS microbeads (Miltenyi-Biotec, Paris, France). For survival data, we included an additional series of 79 patients for a total of 171 patients. These 171 patients were termed in the following Heidelberg-Montpellier (HM) series. According to the Durie-Salmon classification, 15 patients were of stage IA (who progressed into MM requiring therapy), 24 of stage IIA, 2 of stage IIB, 121 of stage IIIA, and 9 of stage IIIB. According to the International Staging System (ISS), 76 patients were of stage I, 66 of stage II, 29 of stage III. 28 patients had IgAK MM, 12 IgAL MM, 64 IgGK MM, 33 IgGL MM, 1 IgDL MM, 18 Bence-Jones kappa MM, 11 Bence-Jones lamda MM, and 4 non-secreting MM. We also used Affymetrix data of a cohort of 345 purified MMC from previously-untreated patients from the Arkansas research group (Little Rock). The patients were treated with total therapy 2 (Barlogie, *et al* 2006) and termed in the following LR-TT2 series. These data are publicly available in the NIH Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE2658. MMC were purified with anti-CD138 MACS microbeads (Miltenyi-Biotec).

For 5 patients with newly-diagnosed MM, bone marrow (BM) T cells (median percentage in BM cells: 8%), monocytes (6% in BM cells) and polymorphonuclear neutrophils (46% in BM cells) were purified as described (Moreaux, *et al* 2005).

Osteoclasts and BM stromal cells (BMSC) obtained from MM patients were generated in vitro as described (Mahtouk, *et al* 2005; Moreaux, *et al* 2005).

Normal BM plasma cells (BMPC) were obtained from healthy donors after informed consent was given. Plasma cells were purified with anti-CD138 MACS microbeads. CD27⁺ memory B cells (MBC) and polyclonal plasmablasts (PPC, CD38⁺⁺, CD20⁻) were obtained and generated in vitro from purified CD19⁺ peripheral blood B cells obtained from healthy donors as previously described (Tarte, *et al* 2002). Normal testis RNA samples were purchased from CliniSciences (Montrouge, France).

Microarray hybridization

RNA was extracted with the RNeasy Kit (Qiagen, Valencia, CA, USA), the SV-total RNA extraction kit (Promega, Mannheim, Germany) and Trizol (Invitrogen, Karlsruhe, Germany) in accordance to the manufacturer's instructions as indicated (De Vos, *et al* 2001). Biotinylated complementary RNA (cRNA) was obtained by double in-vitro transcription, according to the Affymetrix small sample labeling protocol (Affymetrix, Santa Clara, CA, USA). The biotinylated cRNA was fragmented and hybridized to the human U133 A and B (48 patients) and U133 Plus 2.0 GeneChip (123 patients) microarrays according to the manufacturer's instructions (Affymetrix). Fluorescence intensities were quantified and analyzed using the GECOS software (Affymetrix). Arrays were scaled to an average intensity of 100. A threshold of 1 was assigned to values under 1. When different probe sets were available for a same gene, we chose the probe set yielding the highest variation coefficient.

Gene expression profiling (GEP) analysis

In the Affymetrix U133 Plus 2.0 microarrays, a gene is probed by eleven pairs of perfect-match/mismatch oligonucleotides randomly spread over the chip. The signed rank MAS5 algorithm decides after scanning if the corresponding gene can be

statistically declared present (P call) or absent (A call), and delivers a weighted “fluorescence” signal. Gene expression data were analysed with our bioinformatic platform (RAGE, <http://rage.montp.inserm.fr/>) (Reme, *et al* 2008) and Amazonia (<http://amazonia.montp.inserm.fr/>) (Assou, *et al* 2007). Gene expression was assessed in 92 primary MMC samples, 7 BMPC samples, 6 MBC samples, 7 PPC samples from healthy donors and 5 testis samples with U133 Plus 2.0 microarrays. Hierarchical clustering was performed with the Cluster and Treeview softwares from Eisen (Eisen, *et al* 1998). In order to study the role of genes involved in the extrinsic apoptotic pathway (death receptor family and their ligands), we also determined the GEP of the cells of the BM environment, *i.e.* mesenchymal stem cells (MSC), osteoclasts (OST) obtained from 7 MM patients, and polymorphonuclear cells (CD15⁺ cells), T lymphocytes (CD3⁺ cells) and monocytes (CD14⁺ cells) from five newly-diagnosed patients. We also used the GEP from 79 human normal tissues samples available from Hogenesch’s group on a public database (Su, *et al* 2004). These GEP data were determined with Affymetrix U133A and custom-designed GNFH1 arrays. The same global scaling and normalization was used for these and our data.

Real-time RT-PCR analysis

Real-time RT-PCR analysis was done as previously described (Moreaux, *et al* 2004). The assays-on-demand primers and probes and the TaqMan Universal Master Mix were used according to the manufacturer’s instructions (Applied Biosystems, Courtaboeuf, France). Real-time RT-PCR was performed using the ABI Prism 7000 Sequence Detection System and normalized to *β2-microglobulin* for each sample, and compared with the values obtained for a known positive control using the following formula $100/2^{\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct \text{ unknown} - \Delta Ct \text{ positive control}$.

Statistical analysis

Statistical comparisons were made with the non-parametric Mann-Whitney test. *P*-values < .05 were considered as significant. To cluster the samples according to the similarity of their gene expression patterns, we performed an unsupervised principal component analysis (PCA) with our RAGE bioinformatics platform (<http://rage.montp.inserm.fr/>). The statistical significance of differences in event free and overall survivals between groups of patients was estimated by the log-rank test. For survival analysis, we used as a test group our MM series of 171 previously-untreated patients whose MMC GEP were determined with U133 Affymetrix microarrays. We also used as a validation group the independent LR-TT2 series of 345 previously-untreated patients from the Arkansas group whose GEP data, performed with U133 Affymetrix microarrays, are publicly available in the NIH Gene Expression Omnibus (GEO) (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE2658.

Results

The use of Affymetrix microarrays to investigate the various pathways involved in survival/apoptosis of normal B cells, normal and tumor plasma cells.

We investigated the expression of genes coding for molecules involved in the extrinsic and intrinsic apoptotic pathways using Affymetrix microarrays. A consensus-list of genes associated to apoptosis (Table I) was obtained by review of Medline and comprises 58 genes: death receptors and their ligands (Fas/Fas-ligand, TRAIL receptors and decoy receptors for TRAIL), 22 members of the Bcl-2 family, 11 members of caspase family, 7 members of the inhibitors of apoptosis (IAP) family, as well as other proteins involved in mitochondrial apoptosis.

Death receptor family

Fas and Fas ligand

FAS expression is higher in PPC compared to MBC and BMPC and is significantly lower in MMC compared to PPC or BMPC (Figs 2 and S1, Table II). 53% of MM cell samples show a “present” call (Fig S1). Thus, malignant plasma cells either have an absent *FAS* gene call or have a decreased expression compared to plasmablasts or normal plasma cells. *FAS* is highly expressed in the bone marrow environment of patients. Regarding Fas ligand (*FASLG*), the gene is absent in normal B and plasma cells as well as in MMC (Figs 2 and S1). *FASLG* is expressed in CD3⁺ T cells and is absent in polymorphonuclear cells, monocytes, osteoclasts and MSC.

TRAIL receptors, decoy receptors and TRAIL

The two functional TRAIL receptors (*TNFRSF10A* and *TNFRSF10B*) have a higher expression in MBC compared to PPC, BMPC and MMC (Figs 2 and S1, Table II). *TNFRSF10A* and *TNFRSF10B* both are expressed by the majority of MMC-samples (71% and 98%, respectively). Regarding the TRAIL decoy receptors *TNFRSF10C*

and TNFRSF10D, *TNFRSF10C* is absent in all normal and malignant plasma cells. Noteworthy, *TNFRSF10C* is highly expressed by BM polymorphonuclear cells. *TNFRSF10D* gene is present on 89% of MMC. *TNFSF10/TRAIL* is weakly expressed in MBC compared to PPC, BMPC or MMC ($P \leq .01$). The gene is expressed in the majority of primary MMC and has a high expression in 20 % of MMC samples. *TNFSF10* is also highly expressed by bone marrow polymorphonuclear cells and monocytes. Osteoprotegerin (*TNFRSF11B*), which is a soluble decoy receptor for TRAIL, is highly expressed by MSC.

The *TNFRSF1A* gene is expressed at a high level by environment cells (MSC, OST, CD14⁺ and CD15⁺ cells), but it is neither expressed by MBC, normal nor malignant plasma cells (Figs 2 and S1).

FADD, as adapter molecule for the death receptor family allows the recruitment and activation of caspase 8 upon activation of death receptors (Fig 1). *FADD* expression is high in normal PPC that rapidly undergo apoptosis (Tarte, *et al* 2002) compared to MBC, BMPC and MMC samples (ratio ≥ 2.2 ; $P < .003$) (Figs 2 and S1, Table II). *CFLAR/FLIP*, an inhibitor of caspase 8 activation, is expressed by all samples, but lower in MBC compared to normal and tumor plasma cells ($P < .01$, Figs 2 and S1).

The Bcl-2 family

Genes coding for anti-apoptotic proteins

The expression of the 6 known anti-apoptotic members (Bcl-2, Bcl-xL, Mcl-1, Bcl-w, Bfl1/A1 and Bcl-B) can be evaluated with Affymetrix microarrays (Figs 3 and S2). *BCL2L10/BCL-B* was absent in all samples. This probe set works as *BCL2L10* is highly expressed in oocytes (data not shown) (Assou, *et al* 2006). *BCL2L2/BCL-W* is weakly expressed in all samples and there was no present call for this probe set in 54 % of MMC samples.

As shown in Fig S2, four genes - *BCL2*, *BCL2L1*, *MCL1*, *BCL2A1* - showed a “present” call in the majority of samples of a given cell category: *BCL2* and *MCL1* expressions are significantly higher in normal or malignant plasma cells compared to PPC and MBC ($P \leq .03$, Table II). *BCL2L1* expression is higher in plasmablasts and normal or malignant plasma cells compared to MBC ($P \leq .003$). *BCL2A1* is weaker expressed in early and late normal and malignant plasma cells compared to MBC (Table II). This fits well with the known repression of BFL1/A1 by the PRDI-BF1/Blimp1 plasma cell transcription factor (Calame, *et al* 2003; Tarte, *et al* 2004).

Genes coding for pro-apoptotic proteins

The 16 pro-apoptotic protein genes reported in Table I could be interrogated with Affymetrix microarrays (Figs 3 and S3). *BOK* and *BCL2L14* are truly absent in all B and plasma cell samples because they are expressed on testis samples (data not shown). There was no present call for *BAX*, *BAD*, *BIK*, *BBC3* and *BMF* probe sets in the majority of B cell lineage samples and other numerous cell types available within the Hogenesch database (Su, *et al* 2004), suggesting that there is a defect in the probe sets (data not shown). This is likely the case for *BAX*, *BAD*, *BIK* probe sets whose gene and protein expression have been already documented in MM (Egle, *et al* 1997; Jourdan, *et al* 2000; Renner, *et al* 2000; Spets, *et al* 2002).

The overall message is that *BCL2L11*, *BNIP3L* and *BCL2L13* are expressed by all MBC, PPC, BMPC and MMC without major difference in expression level between these populations. *BID* is overexpressed in normal and malignant plasma cells compared to MBC and PPC ($P \leq .003$). Bid thereby constitutes a link between extrinsic and intrinsic apoptosis (Fig 1), thus a high expression of Bid could contribute to apoptosis of MMC induced by activation of the death receptors. Interestingly, *BNIP3* is variably expressed in MMC but is significantly decreased in MMC compared

to BMPC (Table II). 31% of MM samples do not express the gene. *BAK1* and *BCL2L12* have a present call in respectively 20% and 89% of MMC. They are both increased in PPC compared to BMPC and MMC as *BNIP3* ($P < .013$). Both *HRK* and *PMAIP1* are heterogeneously expressed in MMC. Expression of *PMAIP1* is higher in MBC than in normal or malignant plasma cells (Table II).

APAF1 and mitochondrial mediators of apoptosis

As shown in Fig 1, several proteins are involved in the control of the mitochondrial apoptosis pathway. The expression of these genes is shown in Figs 3 and S4. *APAF1*, a critical mediator of caspase 9 activation, was lower expressed in primary MMC compared to MBC, PPC and BMPC (Table II). *CYCS*, *DIABLO* or *HTRA2* genes were expressed in all normal or malignant plasma cells as well as in PPC or MBC (Figs 3 and S4). *AIFM1* expression was significantly increased in PPC compared to MBC, BMPC and MMC (Table II).

Caspase gene expression

11 caspase genes can be interrogated with Affymetrix microarrays (Figs 3 and S5). Except for *CASP5* and *CASP14*, these genes are expressed in at least one B or plasma cell sample type. Of interest, the initiator *CASP8* and *CASP9* are higher expressed in MBC compared to normal or malignant plasma cells (Table II). To the contrary, there is no present call for *CASP10* gene in MBC. *CASP2*, *CASP3*, *CASP6* (Table II) and *CASP7* are increased in PPC compared to MBC, BMPC and MMC ($P < .01$). *CASP5* is absent in all B cell lineage samples but is present in myeloid samples (data not shown).

Inhibitors of apoptosis (IAP/BIRC family)

Affymetrix microarrays make it possible to interrogate 6 members of the IAP family, known to block caspase activity (Figs 3 and S6). *BIRC5/SURVIVIN* is highly

expressed in PPC and lacking in MBC and BMPC (Table II). It is present in 26% of primary MMC in our HM series and in 12% of patients in the LR-TT2 series. *BIRC2/CIAP1*, *XIAP/BIRC4* and *BIRC6/APOLLON* are highly expressed in all MBC-, PPC- and BMPC-samples as well as MMC. *NAIP/BIRC1* is weakly expressed and has an absent call in the majority of samples from B cell lineage, in contrast to CD14, CD15 or osteoclast-samples, which have a higher expression (data not shown). *BIRC3/CIAP2* is not expressed in PPC, highly expressed in BMPC (Table II) and its expression is very heterogeneous in MMC.

Validation of Affymetrix data by real-time RT-PCR

Affymetrix data for 5 deregulated MM genes (*FAS*, *TNFSF10*, *BNIP3*, *CASP2*, *BIRC5*) were validated by real-time RT-PCR using RNA of 3 BMC, 4 PPC, 3 BMPC and 7 MMC samples. Data are shown in Fig 4 and indicate a significant correlation between real-time RT-PCR data and Affymetrix signals ($P \leq .02$).

Principal component analysis of normal and malignant plasma cell samples using Affymetrix probe sets linked with survival/apoptosis

The analysis above makes it possible to select 40 out of the 58 Affymetrix probe sets interrogating the pathways regulating extrinsic and intrinsic apoptosis that are expressed in at least one B- or plasma-cell lineage population (normal MBC, PPC, BMPC and MMC). We consecutively performed a principal component analysis (PCA) of the gene expression of these 40 probe sets with MBC, PPC and BMPC (Fig 5A). The first two principal components (PCA1 and PCA2) accounted for 56% and 21% of the sample variance, respectively. Of interest, these 40 probe sets made it possible to separate MBC, PPC and BMPC in 3 homogeneous clusters. In the figure, the lines represent the contribution of each of the 40 probe sets to design the cell clusters in the PCA data space. Each probe set is represented by a line from the

origin whose direction indicates which cluster or region is most contributed to and length indicates the strength of this contribution (*i.e.* the correlation coefficient of the probe set with the theoretical probe set built by PCA analysis). For 13 probe sets, *e.g.* *TNFRSF10D*, *CASP1*, *CASP4*, *CASP10*, *BCL2L1*, *BAK1*, *BNIP3*, *BCL2L12*, *BIRC2*, *CYCS*, *APAF1*, *DIABLO* and *HTRA2*, the “probe set line” is at a comparable distance to two clusters, indicating a contribution to both clusters. For the remaining 27 probe sets defining only one cluster, the correlation coefficient ranged from 0.3 to 1, of which 18 probe sets (listed in Fig. 5A) had a correlation coefficient higher than 0.8. According to the ranking of the correlation coefficients that indicates the input of a probe set to characterize a cell cluster, the MBC cluster is characterized by *TNFRSF10B*, *PMAIP1*, *TNFRSF10A*, *BCL2A1*, *CASP8* and *CASP9*, the PPC cluster by *CASP3*, *BIRC5*, *AIFM1*, *CASP6*, *FADD*, *FAS* and *CASP2*, the BMPC cluster by *BIRC3*, *MCL1*, *BCL2*, *XIAP* and *BID*. This perfectly fits the analysis above describing the expression of individual probe set in these 3 B and plasma cell populations of healthy individuals (Figs 2 and 3).

Running a PCA of these 40 probe sets on normal and malignant samples (Fig 5B), the two axes represent 18% and 16% of the variance respectively. PPC samples are grouped on the left PCA1 axis due to the expression of *BIRC5*, *CASP2*, *CASP3*, *CASP6*, *FADD* and *AIFM1* genes. Of note, 2 of these genes had adverse prognosis value (see below). MBC and BMPC had a near zero PCA1 value. 51/92 MMC samples had a positive PCA1 value and 41/92 a negative one. The positive PCA1 abscissa is mainly driven by 2 genes, *BCL2* and *BIRC3*. Regarding PCA2 component accounting for 16% of the variance, all normal samples (MBC, BMPC and PPC) have a negative PCA2 ordinate. 61/92 of MMC samples had a positive PCA2 ordinate and 31/92 a negative one, indicating the heterogeneity of MMC samples. The “normal”

PCA2 cluster was driven by *FAS*, *BNIP3* and *APAF1* expression. The MMC cluster (positive PCA2 axis) was driven mainly by *TNFSF10*, in agreement with increased *TNFSF10* expression in MMC as reported above. Clustering of patients into PCA1+ and PCA1- groups or PCA2+ or PCA2- groups did not show prognostic value.

Prognostic value of the probesets

The data reported above indicated that, out of the 58 genes coding for anti- or pro-apoptotic pathways that can be interrogated by Affymetrix microarrays, 40 provide useful information in normal or malignant plasma cells. The expression of 2 of the 40 genes in MMC - *CASP2* and *BIRC5/SURVIVIN* - had prognostic value for event free survival and overall survival in 2 independent series of patients treated with high dose chemotherapy and autologous stem cell transplantation. Of note, these 2 genes are plasmablastic genes, overexpressed in PPC. Patients with a *CASP2* expression in MMC \geq median value (*i.e.* *CASP2*^{high} MMC) have a shorter median event-free survival (EFS, 729 days in HM series and 1443 days in LR-TT2 series) compared with patients *with* *CASP2*^{low} MMC (1326 days in HM series and 1898 days in LR-TT2 series) ($P = .005$ and $P = .019$, respectively) (Figs 6A and 6B). However, the overall survival (OAS) is significantly different in *CASP2*^{high} versus *CASP2*^{low} patients, in the LR-TT2 series only ($P = .015$, data not shown). The second poor prognosis gene, *BIRC5*, encode for survivin that is also involved in cell cycle regulation. Patients with a present call for the *BIRC5* probe set had adverse prognosis in the 2 independent patient series (Figs 6C and 6D; $P = .015$ and $P = 4.10^{-7}$ respectively).

Discussion

The aim of this work was to investigate the gene expression of the main proteins involved in the apoptotic/survival pathways in MMC compared to their expression in normal B cells, normal plasmablastic and plasma cells.

Regarding the extrinsic apoptotic pathway, an elevated expression of *TNFSF10/TRAIL* and *TRAIL* receptors in MMC is observed suggesting an autocrine apoptotic loop could occur. Likely, this apoptotic loop is blocked by decoy receptors. In particular, *TNFRSF10D* is highly expressed by MMC. Osteoprotegerin that is highly expressed by MSC also acts as a soluble decoy receptor and may block TRAIL induced MMC apoptosis (Shipman and Croucher 2003). These TRAIL decoy receptors could hamper the use of TRAIL in MM therapy suggested by several authors (Gazitt 1999; Mitsiades, *et al* 2001). Of interest, agonist antibodies to TRAIL receptors induced MMC apoptosis and could bypass the inhibition of TRAIL-induced apoptosis by decoy receptors as soluble osteoprotegerin (Menoret, *et al* 2006; Locklin, *et al* 2007). An elevated expression of *CFLAR/FLIP* in MMC samples could also confer resistance to apoptosis induced by activation of the extrinsic apoptotic pathway. Indeed, Mitsiades *et al.* (Mitsiades, *et al* 2002) have reported that MMC resistance to TRAIL to be associated with a low pro-caspase 8/Flip ratio although these results remain controversial (Spencer, *et al* 2002).

Among the Bcl-2 family members, we did not observe a significant difference in *MCL1* gene expression, although Mcl-1 protein has been shown to be overexpressed in MMC compared to BMPC (Wuilleme-Toumi, *et al* 2005). This discrepancy could be explained by a high and complex post-translational regulation level of Mcl-1 in MM (Le Gouill, *et al* 2004). The significant decrease of *BNIP3* expression in MMC compared to normal plasma cells is in agreement with a study showing an aberrant

DNA methylation in the region around the *BNIP3* transcription start site, associated with the silencing of the gene in primary MMC from three out of 14 patients with MM (21%) (Murai, *et al* 2005).

Regarding the Durie Salmon stage of the patients with MM, only 3 genes out of 40 genes studied were differentially expressed: *CASP2*, *CASP6* and *BIRC5* (data not shown). The expression of the 3 genes were higher in stage II or III than in stage I MM patients ($P \leq .033$, $P \leq .044$ and $P \leq .018$ respectively). Noteworthy, this result is in agreement with the poor prognosis value of *CASP2* and *BIRC5* in the 2 independent series of patients. The poor prognosis value of *CASP2* encoding gene can be explained by the non-apoptotic functions mediated by the caspase recruitment domain (CARD) in the pro-domain of caspase 2, in particular, by the recruitment of TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein 1 (RIP1) in a caspase 2 complex capable to induce activation of NF- κ B (Lamkanfi, *et al* 2005). Recently, survivin expression has been shown to be correlated to MM progression (Romagnoli, *et al* 2007) and to a poor outcome of patients with multidrug-resistant 1 protein increase after chemotherapy (Nakagawa, *et al* 2006).

In conclusion, this study provides a general view of the contribution of the expression of genes in delineating normal and malignant plasma cells. Out of 40 contributive genes encoding for the major proteins involved in the extrinsic and intrinsic apoptosis pathways, 27 efficiently separate normal memory B cells, plasmablastic cells and bone marrow plasma cells. A high expression of *TNFSF10* delineates a malignant cluster comprising about 50% of the patients. Out of the 40 genes, 2 genes – *CASP2* and *BIRC5/SURVIVIN* – had poor prognosis in 2 independent patients' series and these 2 genes are overexpressed in normal plasmablastic cells.

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Gene symbol	Alias	Probe set	Genes symbol	Alias	Probe set
Extrinsic apoptosis pathway			Mitochondrial apoptosis pathway		
<i>FAS</i>	<i>TNFRSF6</i>	204780_s_at	Bcl-2 family		
<i>FASLG</i>	<i>FASL/TNFSF6</i>	210865_at	Antiapoptotic members		
<i>TNFRSF10A</i>	<i>TRAILR1/DR4</i>	231775_at	<i>BCL2</i>	<i>Bcl-2</i>	203685_at
<i>TNFRSF10B</i>	<i>TRAILR2/DR5</i>	209295_at	<i>MCL1</i>	<i>Mcl-1</i>	200798_x_at
<i>TNFRSF10C</i>	<i>DCR1</i>	206222_at	<i>BCL2L1</i>	<i>BCLX; Bcl-x</i>	212312_at
<i>TNFRSF10D</i>	<i>DCR2</i>	227345_at	<i>BCL2L2</i>	<i>BCL-W; Bcl-w</i>	209311_at
<i>TNFRSF11B</i>	<i>Osteoprotegerin</i>	204932_at	<i>BCL2A1</i>	<i>BFL1/A1</i>	205681_at
<i>TNFSF10</i>	<i>TRAIL</i>	202688_at	<i>BCL2L10</i>	<i>BCL-B; Bcl-B</i>	236491_at
<i>TNFRSF1A</i>	<i>TNFR1</i>	207643_s_at	Proapoptotic members		
<i>TNF</i>	<i>TNF-ALPHA</i>	207113_s_at	BH Multidomain members		
<i>FADD</i>		202535_at	<i>BAX</i>		211833_s_at
<i>CFLAR</i>	<i>FLIP</i>	211862_x_at	<i>BAK1</i>	<i>BAK</i>	203728_at
Caspases			<i>BOK</i>		223349_s_at
<i>CASP1</i>	<i>CASPASE 1</i>	211366_x_at	BH3-only members		
<i>CASP2</i>	<i>CASPASE 2</i>	226032_at	<i>BID</i>		227143_s_at
<i>CASP3</i>	<i>CASPASE 3</i>	202763_at	<i>BCL2L11</i>	<i>BIM</i>	225606_at
<i>CASP4</i>	<i>CASPASE 4</i>	213596_at	<i>BMF</i>		226530_at
<i>CASP5</i>	<i>CASPASE 5</i>	207500_at	<i>BAD</i>		232660_at
<i>CASP6</i>	<i>CASPASE 6</i>	209790_s_at	<i>BIK</i>		205780_at
<i>CASP7</i>	<i>CASPASE 7</i>	207181_s_at	<i>HRK</i>		206864_s_at
<i>CASP8</i>	<i>CASPASE 8</i>	213373_s_at	<i>PMAIP1</i>	<i>NOXA</i>	204285_s_at
<i>CASP9</i>	<i>CASPASE 9</i>	203984_s_at	<i>BNIP3</i>		201849_at
<i>CASP10</i>	<i>CASPASE 10</i>	205467_at	<i>BNIP3L</i>		221479_s_at
<i>CASP14</i>	<i>CASPASE 14</i>	231722_at	<i>BCL2L14</i>	<i>BCLG; Bcl-G</i>	221241_s_at
IAPs family			<i>BBC3</i>	<i>PUMA</i>	211692_s_at
<i>NAIP</i>	<i>BIRC1</i>	204861_s_at	<i>BCL2L12</i>		233110_s_at
<i>BIRC2</i>	<i>CIAP1</i>	202076_at	<i>BCL2L13</i>	<i>BCL-RAMBO</i>	217955_at
<i>BIRC3</i>	<i>CIAP2</i>	210538_s_at	Others proteins		
<i>XIAP</i>	<i>BIRC4</i>	228363_at	<i>APAF1</i>		204859_s_at
<i>BIRC5</i>	<i>SURVIVIN</i>	202095_s_at	<i>CYCS</i>	<i>Cytochrome C</i>	208905_at
<i>BIRC6</i>	<i>APOLLON</i>	233093_s_at	<i>DIABLO</i>	<i>SMAC/DIABLO</i>	219350_s_at
<i>BIRC7</i>	<i>LIVIN</i>	220451_s_at	<i>HTRA2</i>	<i>OMI; PRSS25</i>	211152_s_at
			<i>AIFM1</i>	<i>AIF; PDCD8</i>	205512_s_at
			<i>ENDOG</i>	<i>Endonuclease G</i>	204824_at

Table I. The 58 genes coding for proteins involved in apoptosis regulation and their corresponding Affymetrix probe sets.

Genes overexpressed in MBC versus:	Ratio MBC/PPC	Ratio MBC/BMPC	Ratio MBC/MMC
<i>TNFRSF10A</i>	3.2 <i>P</i> = .004	6.1 <i>P</i> = .003	5.2 <i>P</i> < .001
<i>TNFRSF10B</i>	4.3 <i>P</i> = .003	2.1 <i>P</i> = .007	1.8 <i>P</i> = .007
<i>BCL2A1</i>	7.8 <i>P</i> = .003	2.4 <i>P</i> = .007	3.9 <i>P</i> < .001
<i>CASP8</i>	1.9 <i>P</i> = .010	2.4 <i>P</i> = .002	2.3 <i>P</i> < .001
<i>CASP9</i>	2.2 <i>P</i> = .004	3.3 <i>P</i> = .001	2.7 <i>P</i> < .001
<i>PMAIP1</i>	5.6 <i>P</i> = .003	2.4 <i>P</i> = .003	2.9 <i>P</i> < .001
Genes overexpressed in PPC versus:	Ratio PPC/MBC	Ratio PPC/BMPC	Ratio PPC/MMC
<i>FAS</i>	3.0 <i>P</i> = .003	3.1 <i>P</i> = .002	6.4 <i>P</i> < .001
<i>FADD</i>	2.2 <i>P</i> = .003	2.6 <i>P</i> = .002	2.4 <i>P</i> < .001
<i>AIFM1</i>	4.0 <i>P</i> = .004	5.7 <i>P</i> = .002	3.1 <i>P</i> < .001
<i>BIRC5</i>	44.8 <i>P</i> = .003	55.9 <i>P</i> = .002	22.2 <i>P</i> < .001
<i>CASP2</i>	2.1 <i>P</i> = .010	2.8 <i>P</i> = .002	2.2 <i>P</i> < .001
<i>CASP3</i>	9.1 <i>P</i> = .003	3.5 <i>P</i> = .002	3.9 <i>P</i> < .001
<i>CASP6</i>	4.9 <i>P</i> = .003	4.6 <i>P</i> = .002	3.8 <i>P</i> < .001
Genes overexpressed in BMPC versus:	Ratio BMPC/MBC	Ratio BMPC/PPC	Ratio BMPC/MMC
<i>BCL2</i>	1.5 <i>P</i> = .046	5.3 <i>P</i> = .002	1.0 NS
<i>MCL1</i>	4.6 <i>P</i> = .003	1.7 <i>P</i> = .025	1.1 NS
<i>BID</i>	2.4 <i>P</i> = .003	2.0 <i>P</i> = .002	1.3 <i>P</i> = .007
<i>BIRC3</i>	1.8 <i>P</i> = .046	11.8 <i>P</i> = .002	1.5 <i>P</i> = .039
<i>XIAP</i>	3.0 <i>P</i> = .003	2.4 <i>P</i> = .003	1.8 <i>P</i> < .001
Genes overexpressed in MMC versus:	Ratio MMC/MBC	Ratio MMC/PPC	Ratio MMC/BMPC
<i>TNFSF10</i>	7.7 <i>P</i> = .001	3.1 NS	2.1 NS
Genes underexpressed in MMC versus:	Ratio MBC/MMC	Ratio PPC/MMC	Ratio BMPC/MMC
<i>FAS</i>	2.1 <i>P</i> = .001	6.4 <i>P</i> < .001	2.0 <i>P</i> = .001
<i>APAF1</i>	4.2 <i>P</i> < .001	5.4 <i>P</i> < .001	1.9 <i>P</i> = .002
<i>BNIP3</i>	1.0 NS	4.5 <i>P</i> < .001	2.8 <i>P</i> < .001

Table II: Genes linked to survival/apoptosis differentially expressed in normal B cells, normal and tumoral plasma cells

The ratios of the mean microarray signals in 6 BMC samples, 7 PPC, 7 BMPC and 92 MMC are indicated. *P* values are indicated. NS means “not significant”

Legends to Figures

Fig 1. Extrinsic and intrinsic apoptotic pathways.

In the extrinsic apoptotic pathway, an activation of death receptors by their ligands induces the recruitment of FADD and the recruitment and activation of an initiator caspase, *i.e.* caspase 8. This multiprotein complex is called the “DISC”. In turn, caspase 8 activates an effector caspase, caspase 3, and consecutively the induced cellular apoptosis. Caspase 8 is likewise able to cleave cytosolic Bid, which is translocated to the mitochondrial membrane and thus constitutes a link between extrinsic and intrinsic apoptotic pathways. In the later, various proapoptotic stimuli induce critical modifications on the mitochondrial outer membrane in the balance between pro and antiapoptotic Bcl-2 family members, inducing the membrane-permeabilization and release of cytochrome c into the cytosol. Cytochrome c activates Apaf-1 to constitute a macromolecular complex (apoptosome) which recruits and activates caspase 9 leading to caspase 3 activation and to the proteolysis of target proteins and finally to apoptosis. AIF and endonuclease G are apoptogenic proteins released in the cytosol as cytochrome c but which induce a caspase-independent apoptosis.

Fig 2. Gene expression of death receptor family in normal B cells, normal and tumor plasma cells and bone marrow environment cells.

HG U133 Plus 2.0 Affymetrix microarrays were used to determinate the expression level of 11 genes involved in the extrinsic apoptosis pathway. Histograms show the mean gene signal intensity in 6 memory B cells (MBC), 7 polyclonal plasmablasts (PPC), 7 bone marrow plasma cells (BMPC) obtained from healthy donors, 92

purified myeloma cells (MMC), 7 BM stromal cells (MSC), 7 in vitro-generated osteoclasts (OST) and 5 CD3⁺, CD14⁺ and CD15⁺ cells from newly diagnosed myeloma patients.

Fig 3. Gene expression of Bcl-2 family members, mitochondrial mediators of apoptosis, caspase and IAP family members in normal B cells, normal and tumor plasma cells.

As indicated in Fig 2, histograms show the expression level of genes determined by using HG U133 Plus 2.0 Affymetrix microarrays. Histograms show the mean gene signal intensity in 6 memory B cells (MBC), 7 polyclonal plasmablasts (PPC), 7 BM plasma cells (BMPC) obtained from healthy donors and 92 purified myeloma cells (MMC).

Fig 4. Validation of microarray data.

TNFSF10, *FAS*, *BNIP3*, *CASP2* and *BIRC5* were assayed with real-time RT-PCR in MMC samples. The coefficients of correlation and *P* values for the correlation between Affymetrix and real-time RT-PCR signals were determined with a Spearman test. Black diamonds represent MMC samples (6), open circles are PPC (4), black squares are BMPC (3) and black circles are MBC (3).

Fig 5. PCA of the gene expression of 40 mediators of apoptosis in normal B cells, normal and tumor plasma cells.

(A) Normal B cells (black circles), plasmablast (open circles) and plasma cells (black squares). (B) Normal B cells, normal and malignant plasma cells (black diamonds). The two axes represent the first two principal components (PCA1 and PCA2). The

percentage of the variance of the data accounted by PCA1 and PCA2 is indicated on each axis. In the figure, the lines represent the contribution of each of the 40 probe sets to design the cell clusters in the PCA data space. Each probe set is represented by a line from the origin whose direction indicates which cluster or region is most contributed to and length indicates the strength of this contribution (*i.e.* the correlation coefficient of the probe set with the theoretical probe set built by PCA analysis). The gene name of the major probe sets characterizing a cluster is indicated with the corresponding correlation coefficient in brackets.

Fig 6. Event-free and overall survival of previously untreated MM patients with *CASP2*^{high} or *CASP2*^{low} MMC and *BIRC5*^{present} or *BIRC5*^{absent} MMC.

CASP2 and *BIRC5* gene expressions were assayed in purified MMC with U133 Plus 2.0 microarrays. (A) Event-free survival of MM patients with *CASP2*^{high} or *CASP2*^{low} MMC from HM series (85 versus 85 patients) and (B) from LR-TT2 series (172 versus 172 patients). (C) Overall survival of MM patients with *BIRC5*^{present} or *BIRC5*^{absent} MMC from HM series (45 versus 126 patients) and (D) from LR-TT2 series (41 versus 304 patients). The *P*-value was determined with a log-rank test.

Supporting information

Additional supporting information may be found in the online version of this article.

Fig S1. Detailed gene expression of death receptor family in normal B cells, normal and tumor plasma cells and bone marrow environment cells. Histograms show the gene signal intensity determined with Affymetrix microarrays. Each histogram features an individual sample, MBC (6), PPC (7), BMPC (7) and MMC (92). White histograms mean that the gene is not expressed.

Fig S2. Detailed gene expression of antiapoptotic Bcl-2 family members in normal B cells, normal and tumor plasma cells.

Fig S3. Detailed gene expression of proapoptotic Bcl-2 family members in normal B cells, normal and tumor plasma cells.

Fig S4. Detailed gene expression of mitochondrial mediators of apoptosis.

Fig S5. Detailed gene expression of caspase family members in normal B cells, normal and tumor plasma cells.

Fig S6. Detailed gene expression of IAP family members in normal B cells, normal and tumor plasma cells.