Identification of overexpressed genes in frequently gained/amplified chromosome regions in multiple myeloma

Cristina Largo
Sara Alvarez
Borja Saez
David Blesa
Jose I. Martin-Subero
Ines González-García
Jose A. Brieva
Joaquin Dopazo
Reiner Siebert
María J. Calasanz
Juan C. Cigudosa

Background and Objectives. Multiple myeloma (MM) is a malignancy characterized by clonal expansion of plasma cells. In 50% of the cases, the neoplastic transformation begins with a chromosomal translocation that juxtaposes the *IGH* gene locus to an oncogene. Gene copy number changes are also frequent in MM but less characterized than in other neoplasias. We aimed to characterize genes that are amplified and overexpressed in human myeloma cell lines (HMCL) to provide putative molecular targets for MM therapy.

Design and Methods. Nine HMCL were characterized by fluorescent *in situ* hybridization, comparative genomic hybridization (CGH) and cDNA microarrays for gene expression profiling and copy number changes.

Results. After defining the *IGH*-translocations present in the cell lines, we conducted expression-profiling analysis. Supervised analysis identified 166 genes with significantly different expression among the cell lines harboring *MMSET/FGFR3* (4p16), *MAF* (16q) and *CCND1* (11q13) rearrangements. Array-CGH was then performed. Five chromosomes recurrently affected by gains/amplifications in primary samples and cell lines were analyzed in detail. Sixty amplified and overexpressed genes were found and 25 (42%) of them were only overexpressed when amplified; moreover, six showed a significant association between overexpression and gain/amplification. We also found co-amplification and overexpression for genes located within the same amplicons, such as *MALT1* and *BCL2*.

Interpretation and Conclusions. Parallel analysis of gene copy numbers and expression levels by cDNA microarray in MM allowed efficient identification of genes whose expression levels are elevated because of increased copy number. This is the first time that *MALT1* and *BCL2* have been shown to be overexpressed and amplified in MM.

Key words: multiple myeloma, oncogene, array LGH, expression array.

Haematologica 2006; 91:184-191

From the Cytogenetics Unit, Spanish National Cancer Centre, (CNIO). 28029 Madrid, Spain (CL, SA, DB, JCC); Department of Genetics, University of Navarra, Pamplona, 31008 Spain (BS, MJC); Institute of Human Genetics, University Hospital Schleswig-Holstein, 24105 Kiel, Germany (JIM-S, RS); Immunology Service, "Puerta del Mar" University Hospital, 11009 Cádiz, Spain (IG-G, JAB); Bioinformatics Unit, Spanish National Cancer Centre, (CNIO), 28029 Madrid, Spain (JD).

Correspondence:
Juan C. Cigudosa, Cytogenetics Unit, Spanish National Cancer Centre, (CNIO), C/ Melchor Fernandez Almagro 3, Madrid, 28029, Spain
E-mail: jccigudosa@cnio.es

Multiple myeloma (MM) is a malignancy characterized by a clonal expansion of plasma cells and a wide variability in clinical features, responses to treatment, and survival times among patients.[1,2] Clues to understanding the pathogenesis of myeloma are coming from two directions.[3,4] The first one is chromosomal aberrations, present in virtually all MM, which are either chromosome translocations involving the *IGH* locus and a cellular oncogene; gain and/or losses of chromosomes or specific genomic regions, or a combination of both types of aberrations.[5] The second aspect is the close interaction of the MM cells with bone marrow stromal cells, which is necessary for survival, growth and differentiation of the plasma cells.[4] Understanding of the significance of various translocations in MM has increased, but little is known about the role of gains and losses. Of particular interest is gene overexpression associated with genomic gains or amplifications. These events, frequently observed in other types of cancer, have been used as therapeutic targets. For example, *ERBB2* is overexpressed because of gene amplification in 15 to 20% of breast cancers and represents the target protein for trastuzumab therapy.[6] Therefore, the detection of genes that are gained and overexpressed in MM could eventually offer novel therapeutic approaches. In this work we characterized nine human myeloma cell lines (HMCL) using fluorescence *in situ* hybridization (FISH), conventional comparative genomic hybridization (c-CGH), and cDNA microarrays in order to analyze the gene expression profiles and to determine their relationship with gene copy number changes.

Design and Methods

Human multiple myeloma cell lines

Nine HMCL (RPMI-8226, U-266, OPM-2, SK-MM-2, LP-1, JJN-3, L-636, KARPAS-620, and NCI-H929) were obtained from the DSMZ (Deuche Sammlung von Mikro-

organismen und Zelkuturen GmbH, Braunschweig, Germany) and cultured under recommended conditions. DNA and RNA were extracted using DNAzol® Genomic Isolation Reagent and Tri Reagent® (Molecular Research Cancer Center Inc., Cincinnati, OH, USA), respectively, following the suppliers' protocols. Metaphase cells were prepared by standard cytogenetics methods: mitotic arrest with colcemid (0.1 μg/ mL for 1.5 hours at 37°C; GIBCO, Strachclyde, UK) was followed by hypotonic treatment (75 mM KCl for 15 minutes at 37°C) and fixation with methanol/acetic acid (3:1) before spreading onto slides.

Conventional comparative genomic hybridization

CGH analysis was carried out as previously described with minor modifications.[7] The HMCL genomic test DNA was labeled by CGH-nick translation (Vysis, Chicago, IL, USA) with SpectrumGreen dUTP. Commercially available normal DNA, labeled with Texas Red dUTP, was used as the control reference (Vysis). Images of the hybridized normal male metaphases were analyzed with CytoVision Image Analysis System software (Applied Imaging, Newcastle, UK). Like other researchers,[8] we defined high-level amplification as small regions with a ratio >1.4.

Microarray assays

Microarray assays were performed using the CNIO OncoChip, which contains 7657 different cDNA clones of cancer-related genes (*http://bioinfo.cnio.es/data/oncochip*). For gene expression profiling, 30 μg of the test or reference total mRNA were labeled with fluorescent Cy5-dUTP and Cy3-dUTP (Amersham Biosciences, Cambridge, UK), respectively. Hybridizations were performed at 42°C for 15h as described elsewhere.[9] In all microarray experiments, the reference RNA used was Universal Human RNA (Stratagene, La Jolla, CA, USA). Genes were deemed to be upregulated or downregulated if the ratio was at least two fold. The Gene Expression Profile Analysis Suite (GEPAS) software[10] was used to analyze microarray data. CGH experiments onto cDNA arrays (array-CGH) were performed as described elsewhere previously with minor modifications.[11] Hybridizations and post-hybridization washes were performed at 50°C; slides were scanned in an Agilent Array Scanner (Agilent Technologies, Palo Alto, CA, USA) and quantified using the GenePix Pro 5.0 software (Axon Instruments Inc., Union City, CA, USA). Cy3/Cy5 ratio values were normalized to the median ratio value of all spots in the array. In addition, low quality measurements were excluded from the analysis. Copy gain and loss thresholds were established in control experiments by hybridizing control female DNA against DNA from cell lines containing one to five copies of the X chromosome (*data not shown*). The cut-off values obtained were 0.85 and 1.22 Cy5/Cy3 ratios for loss and gain, respectively. Amplification was considered to be present when ratios exceeded 1.61, meaning gains over five copies. Map positions for arrayed human cDNA clones were assigned according to the July 2003 version of University of California-Santa Cruz Biotechnology Human Genome Working Draft (*http://genome.ucsc.edu/*). In order to identify the gained regions in the array CGH, we used the CGH-Plotter, developed by Autio *et al.*,[12] with our thresholds for gains and losses.

Fluorescence in situ hybridization

FISH assays were carried out as described elsewhere.[13] A multi-FISH assay[14] was used in order to identify the most common reciprocal translocations involving the immunoglobulin heavy and light chain loci. FISH assays were also carried out for three candidate genes: *c-MYC*, *BCL2* and *MALT1*. Probes used for FISH screening were: LSI MYC Dual Colour Break Apart probe (Vysis, Downers Grove, IL, USA), BCL2/IGH probe (Cancer Genetics Inc., Milford MA, USA) and MALT1 Break Apart probe (Master Diagnostic, Granada, Spain). The FISH assays enabled us to determine the presence/absence of translocations involving these genes.

Quantitative real-time polymerase chain reaction assays (qRT-PCR)

First, 1 μg of total RNA was reverse transcribed to cDNA with SuperScript™ II RT (Invitrogen, Carlsbad, CA, USA). Then qRT-PCRT assays (TaqMan®) were performed using primers and probes provided by Assay-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA, USA) for *CCND1* (hs00277039_m1), *CCND2* (hs00153380_m1), *MALT1* (hs01120046_m1), *C-MYC* (hs00153408_m1), and *BCL2* (hs00153350_m1) using the ABI prism 7900 system (Applied Biosystems). For all genes, the *β-actin* gene (hs99999903_m1) was used as a housekeeping gene to normalize the data, as described elsewhere.[15] Total RNA from a pool of tonsil plasma cells, obtained after positive CD138 immunoselection, was used as a control. Tonsil plasma cells were separated according to our previously published protocol.[16]

Results

FISH characterization and global expression profiling

First, the set of HMCL was screened by a customized multi-FISH assay.[14] The assay contained a combination of FISH probes designed to detect the most common reciprocal translocations involving the immunoglobulin heavy and light chain loci. We identified the presence of specific immunoglobulin translocations in all cell lines (Table 1): three showed *CCND1* rearrangements and four showed *FGFR3/MMSET* rearrangements, with SK-MM-2 showing co-existence of *CCND1* and *FGFR3/MMSET* rearrangements. The remaining three lines showed rearrangements of either *MAF* or *MAFB*. These and *FGFR3/MMSET* rearrangements result in *CCND2* upregulation so we performed a qRT-PCR study to evaluate the expression level of *CCND1* and *CCND2*, confirming the expected results (Table 1).

Next, we carried out global expression profiling (GEP) of all cell lines with the CNIO OncoChip, a microarray that contains 7657 cDNA clones representing more than 6000 cancer-related genes. Unsupervised hierarchical clustering to detect possible subgroups within the cell lines failed to segregate subgroups according to immunoglobu-

Table 1. Classification of MM cell lines according to their immunoglobulin (Ig) chromosomal translocation status and their related expression data.

Cell line	Ig locus translocation	FISH confirmed Ig rearranged genes	CCND1 qRT-PCR (a)	CCND2 qRT-PCR (a)	CCND group by expression profiling (b)	TC classification (c)
U-266	ins(11;14)	CCND1	0.248	14.986	D1/D2	11q13
KARPAS-620	t(11;14) t(8;14)	CCND1	0.042	0.521	D1	11q13
SK-MM-2	t(4;14) t(11;14)	FGFR3 MMSET CCND1	0.095	0.004	D1	4p16 11q13
OPM-2	t(4;14)	FGFR3 MMSET	0.000	24.98	D2	4p16
LP-1	t(4;14)	FGFR3 MMSET	0.008	31.338	D2	4p16
NCI-H929	t(4;14)	FGFR3 MMSET	0.299	15.496	D2	4p16
RPMI-8226	t(16;22)	MAF	0.006	18.907	D2	MAF
JJN-3	t(14;16)	MAF	0.001	7.016	D2	MAF
L-363	t(20;22)	MAFB	0.000	16.966	D2	MAF

a: the expression levels of CCND1 and CCND2 were analyzed by qRT-PCR; b: see supplementary Figure 1 for clustering classification after expression profiling into the different CCND subgroups; c: classification of the cell lines in translocation/cyclin D (TC) groups according to the translocation group[18] which includes chromosome rearrangements and CCND expression data.

lin translocation status or cyclin D1 or D2 expression. Expression data were further analyzed by supervised hierarchical clustering.[17] For this purpose, we used a recently proposed MM classification system that integrates data from GEP, the deregulated expression of cyclin D1, D2, or D3, and cytogenetic results.[3,18] Briefly, this system divides MM tumors into eight translocation/cyclin D (TC) groups – 11q13, 6p21, 4p16, maf, D1, D1+D2, D2, and NONE – which appear to be defined by early, and perhaps initiating, oncogenic events. The cell lines investigated in this study fall in three groups: 11q13, 4p16, and maf. SKMM-2 was assigned to the 11q13 group for three reasons: it harbored t(11;14), it overexpressed *CCND1*, and it did not overexpress either *FGFR3* (*not shown*) or *CCND2* (Table 1). We found that 166 genes were differentially expressed (unadjusted $p<0.05$) between these groups (*supplementary data, Figure 1*). Among these genes, *CCND1*, *FGFR3* and *CCR1*, a target of the maf pathway,[19] were found to be differentially expressed in the corresponding subgroups (*supplementary Figure 1*).

Characterization of copy number changes and overexpressed genes

Our purpose was to identify candidate genes that were gained/amplified and overexpressed (A/O genes) as potential targets for therapy. For this purpose, we first performed conventional CGH analysis in the HMCL set. We observed chromosomal imbalances in all of them. The most frequent aberrations were gains in 1q (9/9), 4q (8/9), 7q (8/9), 18q (7/9), 15q (7/9), 11q (7/9) and 8q (7/9) and losses in 13q (9/9), 6q (8/9), 14q(7/9) and 9p (6/9) (*supplementary Figure 2*). High-level amplifications were identified in most frequently gained regions such as 1q21-q32, 7q22-q35, 8q21-q23, 11q14-q25 and 18q11-q22. To obtain information at the gene level, cDNA based array CGH was then performed. This technique enables simultaneous analysis of expression and copy number changes of the genes included in the platform. Thresholds for gain and amplification were established as 1.22 and 1.61, respectively. We focused our analysis on chromosomes 1, 7, 8, 11 and 18, where high-level amplifications and recurrent gains were found by conventional CGH in our cell lines or primary samples from patients.[7,20,21] Using the CGH-plotter software,[12] we identified 13 recurrent gained/amplified regions, some of which covered almost complete chromosome arms (Figure 1A). Within these large regions, we determined some small recurrent subregions with a distinctive level of amplification.

For further analysis, we selected the genes that, within these regions, were gained/amplified and overexpressed 2-fold in at least two of the HMCL (*see Table 2 for a detailed description*). Table 2 shows the 60 genes that fulfil these criteria. The most characteristic gene ontology (GO) term was assigned to each of these genes using the FatiGO web interface.[22] The representative GO terms were related to cell growth and/or maintenance genes (GO:0008151), and genes involved in signal transduction (GO:0007165), cell death (GO:0008219) and regulation of programmed cell death (GO:0043067). Cell death-associated genes, such as *BCL2*, *MALT1*, *DCC*, *ELMO1*, *GAS2* and *BNIP3L*, were significatively overrepresented ($p=0.024$). Furthermore, 25 out the 60 genes (42%) were only overexpressed when gained/amplified (*bolded in Table 2*). Among the 60 recurrently A/O genes, six of them showed a significant association between overexpression and gain/amplification: *CHI3L1*, *ELMO1*, *BNIP3L*, *PLAG1*, *LOC157567*, and *VPS28* (Fisher's test unadjusted-$p<0.05$). Additionally, using these approaches we were able to produces a list of loci deleted and donwregulated in at least two cell lines, in chromosomes 6, 9, 13 and 14 (*Supplementary Table 1*). This additional information provides a more complete picture of both oncogenes and potential tumor supressor genes involved in the pathogenesis of MM.

Analysis of c-MYC, BCL2 and MALT1 expression and amplification status

Finally, we conducted more detailed FISH and qRT-PCR assays on *c-MYC* and *BCL2*, because of their relevance in lymphoid malignancies, and on *MALT1* because of the novelty of its overexpression in MM. Expression levels of these genes in the HMCL were compared with those in total RNA from a pool of tonsil plasma cells obtained after positive CD138 immunoselection. The threshold for overexpression was established as 1.6 fold the value for each gene in the tonsil plasma cells (Table 3).

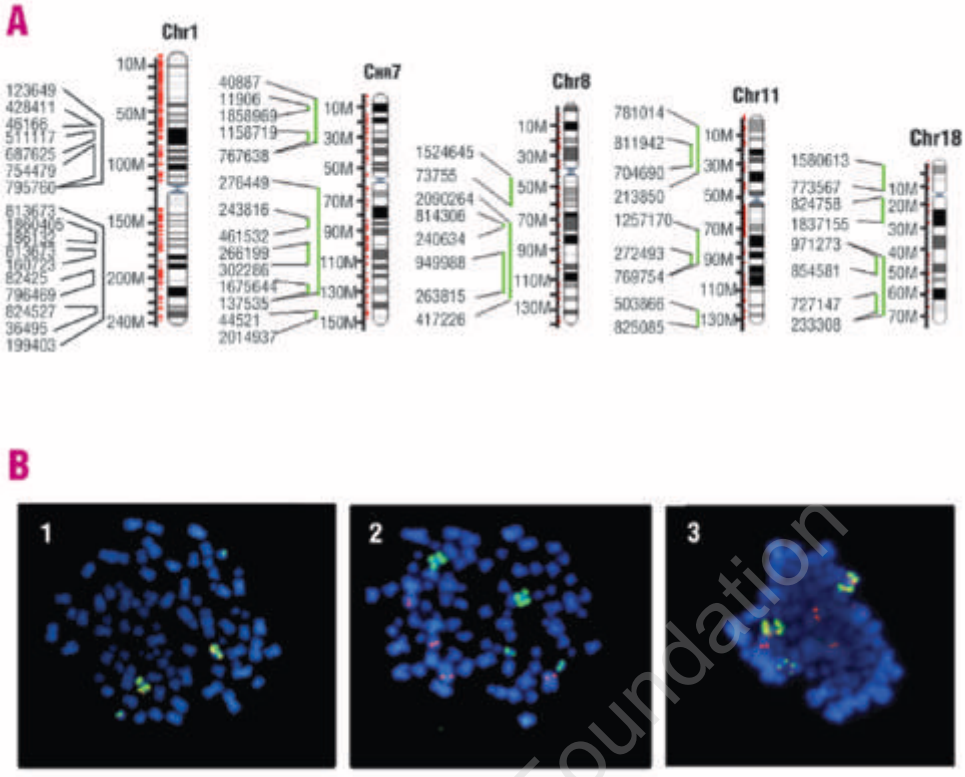

Figure 1. A. Ideograms of chromosomes 1, 7, 8, 11 and 18 representing recurrent gained/amplified regions in nine HMCL. Each chromosome is represented by its band ideogram. Red lines on the left of the idiograms depict the CNIO OncoChip clone coverage in each chromosome. Note that clone coverage is not homogeneous, and some gaps were found. The clone coverage was considered to delimit the gained/amplified regions. Green bars on the left delimit the gained/amplified regions.The limits of the regions indicated as the first and last clones that were included in the gained/amplified region (named by their IMAGE Clone_ID). We also show the minimum region of gain/amplification identified within the largest regions. The figure was created using the InSilico CGH tool, which allows the results of microarray hybridizations to be mapped onto the chromosome coordinates (http://insilicocgh.bioinfo.cnio.es/).[17] Raw expression data and array CGH data are available upon request. **B.** FISH analyses of SK-MM-2 to identify *BCL2* and *MALT1* amplification.1 Metaphase spread showing *MALT1* gene amplification in SK-MM-2. The *MALT1* breakapart probe was designed to include centromeric and telomeric clones flanking the gene, which were differentially labeled with red and green fluorochromes.2 BCL2 amplification is shown using the *BCL2/IGH* probe (Cancer Genetics Inc., Milford MA, USA) with *BCL2* shown in green. The amplified signals are found in the same chromosomes as *MALT1*.3 Co-hybridization of *MALT1* centromeric clones (labeled in red) with the *BCL2/IGH* probe. *MALT1* and *BCL2* are found in the same chromosomes, and also the *IGH* gene is shown in red. FISH assays were carried out as described elsewhere.[13]

c-MYC was overexpressed in four cell lines (as SK-MM-2, LP-1, Karpas-620, OPM-2) that harbored both translocations and gain/amplifications of *c-MYC,* while cell lines with simple translocations (L-363) or gains (JJN-3) failed to show upregulated values (Table 3). FISH analysis revealed that these four HMCL had concomitant amplification/gain and translocation. Regarding genomic changes involving *MALT1*, using a breakapart FISH probe (Figure 1B), we found that seven cell lines showed extra copies of *MALT1* gene and that one of them, the SK-MM-2 line, also had a clear amplification (>10 copies). Expression analysis by qRT-PCR demonstrated *MALT1* overexpression in five HMCL (four with gain/amplification and one with no evidence of genomic copy number changes or translocations involving this locus). Finally, the *BCL2* study revealed, after FISH and qRT-PCR analysis (Table 3), that seven HMCL contained extra copies of the gene with overexpression also present in four of them. FISH data demonstrated that *MALT1* and *BCL2* are both gained in the same seven HMCL but were never found to be translocated in any of the cell lines.

Discussion

We genetically characterized a representative panel of HMCL. Multi-FISH assay identified the presence of at least one specific immunoglobulin translocation in all cell lines (Table 1). After GEP analysis of the HMCL, unsupervised hierarchical clustering failed to segregate subgroups according to either immunoglobulin translocation status or cyclin D1 or D2 expression. We speculated that this could be due to the nature of the cell lines, which mainly represent cases with advanced disease with a high number of chromosomal imbalances which modify the GEP. This pattern of non-clustering is in good concordance with previously observed GEP clusters in primary advanced MM samples which clustered together, but not regarding the translocation involved.[23] Data were further analyzed by supervised hierarchical clustering[17] using translocation/cyclin D groups[18] as the criteria for HMCL classification. Our series of cell lines belonged to three of the groups (11q13, 4p16, and maf) and the supervised

Table 2. Genes gained/amplified (array CGH ratio >1.22) and overexpressed (expression array ratio >2) in at least two of the HMCL analyzed according to microarray assays.

Gene	*Description*	*Cytoband*	*Start*	*% of cell lines with overexpression*	
				Gained[1]	*Not gained*[2]
NFIA[3]	KIAA0485 protein	1p31.3-p31.2	60471035	50% (2/4)	20% (1/5)
LMO4[4]	LIM domain only 4	1p22.3	86963064	50% (2/4)	60% (3/5)
DISC1[4]	Disrupted in schizophrenia 1	1p22	92149953	50% (2/4)	20% (1/5)
EST[4]	IMAGE: 683331	1p22.1	93013822	**50% (2/4)**	**0% (0/5)**
CD53[4]	CD53 antigen	1p31-p12	110282336	100% (2/2)	57% (4/7)
EST1B[4]	Est1p-like protein B	1q22	151984237	100% (2/2)	29% (2/7)
AIM2[3]	Absent in melanoma 2	1q22	154764543	67% (2/3)	50% (3/6)
Ufc1[4]	Ufm1-conjugating enzyme 1	1q23.1	156855810	67% (2/3)	50% (3/6)
CAPON	C-terminal PDZ domain ligand of neuronal nitric oxide synthase	1q23.1-q23.2	157785645	40% (2/5)	25% (1/4)
LOC54499[3]	Putative membrane protein	1q22-q25	161347624	40% (2/5)	50% (2/4)
SELL[3]	Selectin L (lymphocyte adhesion molecule 1)	1q23-q25	165310642	**40% (2/5)**	**0% (0/4)**
PTPN7[3]	Protein tyrosine phosphatase, non-receptor type 7	1q32.1	197550033	50% (2/4)	60% (3/5)
*CHI3L1**[4]	Chitinase 3-like 1 (cartilage glycoprotein-39)	1q32.1	198577702	67% (2/3)	17% (1/6)
GUK1	Guanylate kinase 1	1q32-q41	224066618	50% (2/4)	20% (1/5)
*ELMO1**[4]	Engulfment and cell motility 1 (ced-12 homolog, *C. elegans*)	7p14.1	36540538	**100% (3/3)**	**0% (0/6)**
PURB[3]	Purine-rich element binding protein B	7p13	44562097	100% (2/2)	14% (1/7)
GUSB	Glucuronidase, β	7q21.11	64065460	66% (2/3)	17% (1/6)
PFTK1	PFTAIRE protein kinase 1	7q21.1	88873959	50% (2/4)	40% (2/5)
CDK6[4]	Cyclin-dependent kinase 6	7q21.2	90888487	75% (3/4)	20% (1/5)
TRRAP[3]	Transformation/transcription domain-associated protein	7q22.1	97010689	**50% (2/4)**	**0% (0/5)**
SRPK2	SFRS protein kinase 2	7q22.3	103240523	**40% (2/5)**	**0% (0/4)**
EST[4]	IMAGE:624347	7q22.3	104785450	**40% (2/5)**	**0% (0/4)**
EST	IMAGE:462229	7q31	115393089	33% (2/6)	33% (1/3)
MLL3	B melanoma antigen family, member 4	7q34-q36	150080292	**40% (2/5)**	**0% (0/4)**
*BNIP3L**	BCL2/adenovirus E1B 19kDa interacting protein 3-like	8p21	26594052	**100% (2/2)**	**0% (0/7)**
CEBPD[4]	CCAAT/enhancer binding protein (C/EBP), δ	8q11	48536540	**50% (2/4)**	**0% (0/5)**
LYN	V-yes-1 Yamaguchi sarcoma viral related oncogene homolog	8q12.1	56731374	57% (4/7)	50% (1/2)
*PLAG1**[4]	Pleiomorphic adenoma gene 1	8q12.1	57236036	100% (5/5)	25% (1/4)
GGH	Gamma-glutamyl hydrolase	8q12.1	63867237	22% (2/9)	–
EST	IMAGE:323274	8q13.1	67480390	**50% (4/8)**	**0% (0/1)**
TPD52	Tumor protein D52	8q21	80995889	100% (7/7)	50% (1/2)
FLJ14007	Hypothetical protein FLJ14007	8q21.13	82662132	**43% (3/7)**	**0% (0/2)**
EST	IMAGE: 809828	8q21.2	86086919	33% (2/6)	33% (1/3)
TP53INP1	Tumor protein p53 inducible nuclear protein 1	8q22	95910144	100% (4/4)	80% (4/5)
*LOC157567**	Hypothetical protein LOC157567	8q22.3	101603041	**83% (5/6)**	**0% (0/3)**
CTHRC1	Collagen triple helix repeat containing 1	8q22.3	104452013	**43% (3/7)**	**0% (0/2)**
LRP12	Low density lipoprotein-related protein 12	8q22.2-q23.1	105569698	43% (3/7)	100% (2/2)
EIF3S3	Eukaryotic translation initiation factor 3, subunit 3 gamma, 40kDa	8q24.11	117718260	**25% (2/8)**	**0% (0/1)**
EST	IMAGE:1284498	8q24.12	119375502	**100% (8/8)**	**0% (0/1)**
MRPL13	Mitochondrial ribosomal protein L13	8q24.12	121466001	**25% (2/8)**	**0% (0/1)**
FLJ14825	Hypothetical protein FLJ14825	8q24.13	124290005	**25% (2/8)**	**0% (0/1)**
ZHX1	Zinc fingers and homeoboxes 1	8q24.13	124318757	**25% (2/8)**	**0% (0/1)**
FBXO32	F-box protein 32	8q24.13	124567904	**25% (2/8)**	**0% (0/1)**
MYC	V-myc myelocytomatosis viral oncogene homolog (avian)	8q24.12-q24.13	128836577	**71% (5/7)**	**0% (0/2)**
CYC1[4]	Cytochrome c-1	8q24.3	145286499	100% (2/2)	29% (2/7)
*VPS28**[4]	Vacuolar protein sorting 28 (yeast)	8q24.3	145686880	100% (2/2)	0% (0/7
GAS2	Growth arrest-specific 2	11p14.3-15.2	23497753	75% (3/4)	40% (2/5)
WNT11[4]	Wingless-type MMTV integration site family, member 11	11q13.5	77436402	**100% (3/3)**	**33% (2/6)**
RAB30[3]	RAB30, member RAS oncogene family	11q12-q14	84231298	50% (2/4)	100% (5/5)
PICALM	Phosphatidylinositol binding clathrin assembly protein	11q14	87284417	100% (3/3)	33% (2/6)
PANX1	Pannexin 1	11q14.3	95369855	**40% (2/5)**	**0% (0/4)**
POU2AF1	POU domain, class 2, associating factor 1	11q23.1	112735138	100% (3/3)	83% (5/6)
FLI1	Friend leukemia virus integration 1	11q24.1-q24.3	130076675	**57% (4/7)**	**0% (0/2)**
C18orf8[3]	Chromosome 18 open reading frame 8	18q11.2	20815018	**86% (6/7)**	**0% (0/2)**
KCTD1[4]	Potassium channel tetramerisation domain containing 1	18q12.1	23766411	50% (2/4)	20% (1/5)
DCC	Deleted in colorectal carcinoma	18q21.3	50213955	**43% (3/7)**	**0% (0/2)**
TCF4	Transcription factor 4	18q21.1	52797605	67% (4/6)	67% (2/3)
MALT1	Mucosa associated lymphoid tissue lymphoma translocation gene 1	18q21	56312726	50% (2/4)	60% (3/5)
EST	IMAGE: 845609	18q21.31	56781141	67% (2/3)	33% (2/6)
BCL2	B-cell CLL/lymphoma 2	18q21.3	60763885	100% (6/6)	67% (2/3)

*EST: expressed sequence tags: we provide the IMAGE Clone_ID. [1]Percentage of cell lines that simultaneously present gene overexpression and gain/amplification of the genomic region where the gene is located. [2]Percentage of cell lines that overexpress the gene, but that do not show genomic gain in the region where the gene is located. [3]Genes appearing amplified (array CGH ratio > 1.61) in all cell lines harboring the genomic gain. [4]Genes gained (array CGH ratio > 1.22, <1.61) in all cell lines harboring the genomic gain. *These genes were found to be preferentially overexpressed when gain/amplified (Fisher's test unadjusted p<0.05). Bold lines indicate genes that are only overexpressed when gained/amplified.*

Table 3. Results of microarrays, FISH and qRT-PCR analyses for *C-MYC*, *BCL2* and *MALT1* genes.

Cell line	Ploidy	C-MYC FISH	% of cells with gain	qRT-PCR
SK-MM-2	3	G+T	100	4,82*
U-266	2	N	0	0,06
OPM-2	3	G+T	100	2,26*
RPMI-8226	3	N	0	1,75
JJN 3	2-3	G	87	1,31
L-363	2-3	N+T	0	1,51
LP-1	2-3	A+T	95	5,16*
KARPAS-620	3	A+T	100	2,15*
NCI H-929	2	N/D	100	1,31
Tonsil PC	–	–	–	1,09

Cell line	Ploidy	MALT1 FISH	% of cells with gain	qRT-PCR
SK-MM-2	3	A	100	2,84*
U-266	2	N	8	6,71*
OPM-2	3	G	75	3,05*
RPMI-8226	3	G	58	1,59
JJN 3	2-3	G	98	1,10
L-363	2-3	N	10	1,93
LP-1	2-3	G	89	6,41*
KARPAS-620	3	G	96	2,09*
NCI H-929	2	G	70	1,55
Tonsil PC	–	–	–	1,23

Cell line	Ploidy	BCL2 FISH	% of cells with gain	qRT-PCR
SK-MM-2	3	A	100	62,13*
U-266	2	N	4	18,24*
OPM-2	3	G	88	7,69*
RPMI-8226	3	G	77	4,89
JJN 3	2-3	G	69	2,57
L-363	2-3	N	7	9,40*
LP-1	2-3	G	75	26,46*
KARPAS-620	3	G	98	4,00
NCI H-929	2	G	70	5,34*
Tonsil PC	–	–	–	3,42

*A: amplified, G: gained, N: without changes, D: deleted, T: translocated and NA: not available. PC: plasma cells. In FISH assays a locus was considered as gained when we found at least one copy more than the ploidy level of the cell line and amplified when more than six copies were found. * Overexpressed by qRT-PCR. The overexpression threshold was considered as 1.6-fold the expression level in plasma cells.*

analysis found 166 genes (unadjusted $p<0.05$) differentially expressed between the groups (*supplementary Figure 1*). Among these genes, *CCND1*, *FGFR3* and *CCR1* a target of the maf pathway,[19] were differentially expressed in the corresponding subgroups, as was expected.

In order to identify candidate genes that were gained/amplified and overexpressed cDNA-based array CGH was performed. We have previously performed conventional CGH analysis in the HMCL set. Among all the aberrations identified, high-level amplifications were characterized in most frequently gained regions such as 1q21-q32, 7q22-q35, 8q21-q23, 11q14-q25 and 18q11-q22. These results are in concordance with those from previous studies on primary MM[7,21,24] and cell lines.[25] Continuing research, we focused on five chromosomes (1, 7, 8, 11 and 18) in which high-level amplifications and recurrent gains were found by conventional CGH in our cell lines or primary patient samples.[7,20,21] Thirteen recurrent gained/ amplified regions were identified; some of them spanned almost complete chromosome arms (Figure 1A). Within these regions, some small recurrent sub-regions of amplification were determined. This feature of large regions of gain/amplification including smaller sub-regions of different degrees of genomic imbalances has also been reported for amplicons in some solid tumors such as breast cancer and cell lines.[26,27] We were able to identify 60 genes that were gained/amplified and overexpressed. The gene ontology approach showed that genes with cell death-associated functions, such as *BCL2*, *MALT1*, *DCC*, *ELMO1*, *GAS2* and *BNIP3L*, were significatively overrepresented. Twenty-five out the 60 genes (42%) were only overexpressed when gained/amplified. Although the gained/amplified regions contained a large number of overexpressed genes, especially in cells carrying the gain, other genes were also upregulated in the HMCL without the gain, pointing to alternative mechanisms of overexpression, such as translocations or hypomethylation described for different tumors. Furthermore, six of the amplified and overexpressed genes showed a significant association between overexpression and gain/amplification: *CHI3L1*, *ELMO1*, *BNIP3L*, *PLAG1*, *LOC157567*, and *VPS28* (Fisher's test unadjusted $p<0.05$). Among this group of genes, we mention in particular *ELMO1*, related with cell motility and cell death, and *PLAG1*, overexpressed in epithelial tumors.[28] We characterized 1q gain/amplification but were not able to identify *CKS1B*, a gene that has been shown to be gained in MM patients,[20] as an amplified and overexpressed gene because, unfortunately, this gene was not included in our array experiments. Futher analysis of the gained/amplified and overexpressed genes showed that the genomic mechanism linked with overexpression was exclusively amplification in nine genes and gain in fifteen genes, whereas the remaining were affected by both mechanisms. Consequently, not only were amplifications associated with overexpression, but also low-copy gains had similar outcomes on gene expression, as has been described in other reports.[29,30]

FISH and qRT-PCR assays were carried out on *c-MYC*, *BCL2*, and *MALT1* genes. These genes were selected from the 60 gained/amplified and overexpressed genes previously described because of their relevance in lymphoid malignancies for *c-MYC* and *BCL2*, and because of the novelty of its overexpression in MM for *MALT1*. The gene ontology approach also indicated that cell-death related genes, *BCL2* and *MALT1*, were overrepresented among the gained/amplified and overexpressed genes. The expression levels of these three genes was analyzed by comparison with total RNA from a pool of tonsil plasma cells. In general, data from array CGH and FISH were concordant for most cell lines with some discrepancies that were attributable to the levels of ploidy and oligoclonality. The comparison of expression data from gene

expression profiling and qRT-PCR showed some concordance for most of the cell lines but also some controversial results, mostly for the RPMI-8266, JJN-3, and OPM-2 cell lines. These differences occurred because different reference RNA were used as controls: GEP was done using a commercial RNA pool (Table 1) whereas qRT-PCR was done with a pool of tonsil plasma cells. Because the commercial RNA pool may show a baseline level of expression for some specific genes that does not correspond with the levels of expression in plasma cells, we only discuss the results based on the qRT-PCR data.

Four cell lines showed *c-MYC* overexpression. Those cell lines harbored both translocations and gain/amplifications of *c-MYC*. Nevertheless, we did not find *c-MYC* upregulation in cell lines harboring simple translocations or gains (Table 3). This suggests that either the overexpression of *c-MYC* requires a combination of both mechanisms or that this gene is not the target of the rearrangement. However, high levels of *c-MYC* expression have been associated with more aggressive cases of primary MM.[23] The *MALT1* gene has been found to be recurrently rearranged in chromosomal translocations with *API2* and the *IGH* locus in mucosa-associated lymphoid tissue lymphomas[31] and has also been described to be amplified in B-cell non-Hodgkin's lymphomas.[32] The functions of the MALT1 protein have not been fully characterized, but it has been demonstrated to have a synergic role with CARMA1 and BCL10 in *NF-κB* signaling.[33] We found seven cell lines with extra copies of *MALT1*. qRT-PCR demonstrated *MALT1* overexpression in four of the gained/amplified cases. *BCL2* is a survival factor for many cell types and its overexpression has been described in HMCL and in freshly isolated myeloma cells.[34,35] We also identified seven HMCL with extra copies of *BCL2* and four cell lines showed upregulation. In summary, FISH data demonstrated that, whereas no translocations were identified, both *MALT1* and *BCL2* are gained in the same seven HMCL. For the first time in MM, we demonstrated simultaneous overexpression of *MALT1* and *BCL2* in three out of these seven cell lines. This phenomenon of simultaneous amplification and overexpression of co-localized genes has been reported in breast tumors for *ERBB2*, *GRB7* and *MLN64/CAB1* in the 17q12-q21 amplicon.[11,36] Sanchez-Izquierdo *et al.*[32] reported a patient with gastric MALT lymphoma with *MALT1* and *BCL2* co-amplification but the co-expression could not be further confirmed. In their study they also described 16 lymphoma cell lines with an 18q21 amplified region. They studied the co-existence of *BCL2* and *MALT1* amplification and overexpression and they described that the amplification peak predominantly involved *BCL2* in diffuse large B-cell and mantle cell lymphoma cell lines whereas SSK-41 and Karpas 1718 (marginal zone lymphoma cell lines) displayed the amplification maxima centred around the *MALT1* locus. They found no examples of simultaneous overexpression of both genes. Although no patients were analyzed in this study, the cell lines included represent the major translocation groups that do actually occur in primary MM samples from patients. Parallel analysis of gene copy numbers and expression levels by cDNA microarray in MM allowed efficient identification of genes whose expression levels are elevated because of increased copy number. The information provided here should foster protein expression studies of candidate genes in primary samples to identify putative therapeutic targets.

CL, SA and JCC contributed to the design, analysis, and interpretation of the data. CL and SA wrote the paper, with the final supervision of JCC. BS and JIMS designed the FISH probes, conducted the experiments and interpreted them. CL, SA, DB, and JD were involved in the microarray analyses and their interpretation (including software). IGG and JAB contributed to the extraction and analysis of expression of normal plasma cells. Finally, RS, MJC and JCC contributed to the final revision and critical reading of the manuscript. JCC takes primary responsibility for the paper. Responsibilities for Figure 1: CL, BS, JD; supplementary Figures 1 and 2: CL, DB and SA; Tables 1 and 2: BS and CL; Table 2: CL, DB, JD and SA; Table 3: CL, IGG, JAB and SA; supplementary Table 1: CL, DB, JCC and SA.

We thank M. Carmen Martín for providing technical support. This work was funded by Red Temática FIS G03/136 Mieloma Múltiple y otras ganmapatías: de la Génesis a la Terapeútica and partially supported by a grant from the Fundació La Caixa and Deutsche Krebshilfe. CL is the recipient of a Fellowship from the Gobierno de Navarra. Authors declare that they have no potential conflict of interest.

Manuscript received August 25, 2005. Accepted December 1, 2005.

References

1. Kyle RA, Rajkumar SV. Multiple myeloma. N Engl J Med 2004; 351:1860-73.
2. Sirohi B, Powles R. Multiple myeloma. Lancet 2004;363:875-87.
3. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in biology of multiple myeloma: clinical applications. Blood 2004;104:607-18.
4. Seidl S, Kaufmann H, Drach J. New insights into the pathophysiology of multiple myeloma. Lancet Oncol 2003; 4:557-64.
5. Kuehl WM, Bergsagel PL. Multiple myeloma: evolving genetic events and host interactions. Nat Rev Cancer 2002;2:175-87.
6. Vogel CL, Cobleigh MA, Tripathy D, Gutheil JC, Harris LN, Fehrenbacher L, et al. Efficacy and safety of trastuzumab as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 2002; 20:719-26.
7. Cigudosa JC, Rao PH, Calasanz MJ, Odero MD, Michaeli J, Jhanwar SC, et al. Characterization of nonrandom chromosomal gains and losses in multiple myeloma by comparative genomic hybridization. Blood 1998; 91: 3007-10.
8. Forozan F, Mahlamaki EH, Monni O, Chen Y, Veldman R, Jiang Y, et al. Comparative genomic hybridization analysis of 38 breast cancer cell lines: a basis for interpreting complementary DNA microarray data. Cancer Res 2000;60:4519-25.
9. Tracey L, Villuendas R, Dotor AM, Spiteri I, Ortiz P, Garcia JF, et al. Mycosis fungoides shows concurrent deregulation of multiple genes involved in the TNF signaling pathway: an expression profile study. Blood 2003;102:1042-50.
10. Herrero J, Al-Shahrour F, Diaz-Uriarte R, Mateos A, Vaquerizas JM, Santoyo J, et al. GEPAS: a web-based resource for microarray gene expression data analysis. Nucleic Acids Res 2003;31: 3461-7.
11. Pollack JR, Perou CM, Alizadeh AA, Eisen MB, Pergamenschikov A, Williams CF, et al. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat Genet 1999; 23:41-6.
12. Autio R, Hautaniemi S, Kauraniemi P, Yli-Harja O, Astola J, Wolf M, et al. CGH-Plotter: MATLAB toolbox for CGH-data analysis. Bioinformatics

2003;19:1714-15.
13. Rodriguez-Perales S, Martinez-Ramirez A, de Andres SA, Valle L, Urioste M, Benitez J, et al. Molecular cytogenetic characterization of rhabdomyosarcoma cell lines. Cancer Genet Cytogenet 2004;148:35-43.
14. Karpas A, Harder L, Czepulkowski B, Bloxham D, Saward R, Dremucheva A, et al. Studies of four new human myeloma cell lines. Leuk Lymphoma 2005;46:101-12.
15. Martinez-Delgado B, Melendez B, Cuadros M, Alvarez J, Castrillo JM, Ruiz De La Parte A, et al. Expression profiling of T-cell lymphomas differentiates peripheral and lymphoblastic lymphomas and defines survival related genes. Clin Cancer Res 2004; 10: 4971-82.
16. Medina F, Segundo C, Campos-Caro A, Gonzalez-Garcia I, Brieva JA. The heterogeneity shown by human plasma cells from tonsil, blood, and bone marrow reveals graded stages of increasing maturity, but local profiles of adhesion molecule expression. Blood 2002; 99:2154-61.
17. Herrero J, Vaquerizas JM, Al-Shahrour F, Conde L, Mateos A, Diaz-Uriarte JS, et al. New challenges in gene expression data analysis and the extended GEPAS. Nucleic Acids Res 2004; 32: W485-91.
18. Bergsagel PL, Kuehl WM, Zhan F, Sawyer J, Barlogie B, Shaughnessy J Jr. Cyclin D dysregulation: an early and unifying pathogenic event in multiple myeloma. Blood 2005;106:296-303.
19. Hurt EM, Wiestner A, Rosenwald A, Shaffer AL, Campo E, Grogan T, et al. Overexpression of c-maf is a frequent oncogenic event in multiple myeloma that promotes proliferation and pathological interactions with bone marrow stroma. Cancer Cell 2004;5:191-9.
20. Sawyer JR, Tricot G, Lukacs JL, Binz RL, Tian E, Barlogie B, et al. Genomic instability in multiple myeloma: evidence for jumping segmental duplications of chromosome arm 1q. Genes Chromosomes Cancer 2005;42:95-106.
21. Gutierrez NC, Garcia JL, Hernandez JM, Lumbreras E, Castellanos M, Rasillo A, et al. Prognostic and biological significance of chromosomal imbalances assessed by comparative genomic hybridization in multiple myeloma. Blood 2004;104:2661-6.
22. Al-Shahrour F, Diaz-Uriarte R, Dopazo J. FatiGO: a web tool for finding significant associations of gene ontology terms with groups of genes. Bioinformatics 2004;20:578-80.
23. Mattioli M, Agnelli L, Fabris S, Baldini L, Morabito F, Bicciato S, et al. Gene expression profiling of plasma cell dyscrasias reveals molecular patterns associated with distinct IGH translocations in multiple myeloma. Oncogene 2005;24:2461-73.
24. Gutierrez NC, Hernandez JM, Garcia JL, Canizo MC, Gonzalez M, Hernandez J, et al. Differences in genetic changes between multiple myeloma and plasma cell leukemia demonstrated by comparative genomic hybridization. Leukemia 2001; 15:840-5.
25. Inoue J, Otsuki T, Hirasawa A, Imoto I, Matsuo Y, Shimizu S, et al. Overexpression of PDZK1 within the 1q12-q22 amplicon is likely to be associated with drug-resistance phenotype in multiple myeloma. Am J Pathol 2004; 165:71-81.
26. Ray ME, Yang ZQ, Albertson D, Kleer CG, Washburn JG, Macoska JA, et al. Genomic and expression analysis of the 8p11-12 amplicon in human breast cancer cell lines. Cancer Res 2004; 64: 40-7.
27. Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, et al. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 1998;20:207-11.
28. Astrom AK, Voz ML, Kas K, Roijer E, Wedell B, Mandahl N, et al. Conserved mechanism of PLAG1 activation in salivary gland tumors with and without chromosome 8q12 abnormalities: identification of SII as a new fusion partner gene. Cancer Res 1999;59:918-23.
29. Hyman E, Kauraniemi P, Hautaniemi S, Wolf M, Mousses S, Rozenblum E, et al. Impact of DNA amplification on gene expression patterns in breast cancer. Cancer Res 2002;62:6240-5.
30. Fabris S, Agnelli L, Mattioli M, Baldini L, Ronchetti D, Morabito F, et al. Characterization of oncogene dysregulation in multiple myeloma by combined FISH and DNA microarray analyses. Genes Chromosomes Cancer 2005;42:117-27.
31. Streubel B, Lamprecht A, Dierlamm J, Cerroni L, Stolte M, Ott G, et al. t(14;18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma. Blood 2003;101:2335-9.
32. Sanchez-Izquierdo D, Buchonnet G, Siebert R, Gascoyne RD, Climent J, Karran L, et al. MALT1 is deregulated by both chromosomal translocation and amplification in B-cell non-Hodgkin lymphoma. Blood 2003;101: 4539-46.
33. Lucas PC, Yonezumi M, Inohara N, McAllister-Lucas LM, Abazeed ME, Chen FF, et al. Bcl10 and MALT1, independent targets of chromosomal translocation in MALT lymphoma, cooperate in a novel NF-κB signaling pathway. J Biol Chem 2001; 276: 19012-9.
34. Miguel-Garcia A, Orero T, Matutes E, Carbonell F, Miguel-Sosa A, Linares M, et al. bcl-2 expression in plasma cells from neoplastic gammopathies and reactive plasmacytosis: a comparative study. Haematologica 1998;83:298-304.
35. Pettersson M, Jernberg-Wiklund H, Larsson LG, Sundstrom C, Givol I, Tsujimoto Y, et al. Expression of the bcl-2 gene in human multiple myeloma cell lines and normal plasma cells. Blood 1992;79:495-502.
36. Kauraniemi P, Barlund M, Monni O, Kallioniemi A. New amplified and highly expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA microarrays. Cancer Res 2001;61:8235-40.