Molecular and biological characterization of three novel interleukin-6-dependent human myeloma cell lines

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Background and Objectives. Established human myeloma cell lines (HMCL) have significantly contributed to the investigation of the biological aspects of multiple myeloma. Our study reports the molecular and biological characterization of three novel interleukin-6 (IL-6)-dependent HMCL (CMA-01, CMA-02, CMA-03) established from the malignant plasma cells of myeloma patients with extramedullary disease.

Design and Methods. The immunophenotype, cell growth characteristics, IL-6 pathway, chromosomal alterations and gene expression profiles of the three HMCL were investigated.

Results. The plasma cell origin of the three Epstein-Barr virus-negative HMCL was confirmed by immunophenotypic analysis. Cytogenetic and fluorescence *in situ* hybridization analyses revealed the presence of complex karyotypes with many numerical and structural chromosomal abnormalities. All three HMCL are positive for the t(8;14); CMA-01 and CMA-02 showed t(11;14) and t(14;16) translocations, respectively. The three HMCL grow slowly at a relatively low saturation density and depend on exogenous IL-6 for their survival and proliferation. The comparison of the gene expression profiles of the three HMCL versus those of the purified tumor plasma cells from which the cell lines were derived identified a set of differentially expressed genes mainly involved in the cell proliferation pathway.

Interpretations and Conclusions. Extensively characterized large HMCL panels that reflect the heterogeneity of the disease may improve our understanding of the pathogenetic events and clinical progression of multiple myeloma.

Key words: multiple myeloma; human myeloma cell lines; IL-6-dependent cell lines

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Multiple myeloma is a malignant proliferation of bone marrow plasma cells characterized by marked genomic instability, highly heterogeneous genetic lesions, and a varied clinical course.[1,2] The survival and proliferation of normal and malignant plasma cells strictly depend on a complex interaction with the bone marrow microenvironment mediated by adhesion molecules and the production of several cytokines, particularly interleukin-6 (IL-6).[3] Over the years, established human myeloma cell lines (HMCL) have significantly contributed to the investigation of the biological aspects of multiple myeloma, such as the production of and response to growth factors, the involvement in angiogenic processes, and the resistance to chemotherapeutic agents.[4] A number of chromosome translocations affecting the immunoglobulin loci in plasma cell dyscrasias have been identified by means of the molecular analysis of HMCL; particularly, the t(11;14)(q13;q32), t(4;14)(p16.3;q32), t(6;14)(p21.1;q32), t(14;16)(q32;q23), t(14;20)(q32;q11) and t(8;14)(q24;q32), which lead to the deregulation of their respective target genes *CCND1, FGFR3* and *MMSET, CCND3, MAF, MAFB*, and *MYC*.[5] In this respect, it is conceivable that large HMCL panels reflecting the heterogeneity of the disease may improve our understanding of the pathogenetic events and clinical progression of multiple myeloma, and may help to identify specific therapeutic targets.

The slow growth rate of plasma cells, the need for specific growth factors critical for the development of multiple myeloma *in vivo*, and the overgrowth of Epstein-Barr virus (EBV)-infected B-lymphoblastoid cells possibly present in the original samples are all limiting factors when establishing HMCL. Most HMCL have been

Table 1. Clinical characteristics of the patients.

	PCL-01 (CMA-01)	*PCL-02 (CMA-02)*	*PCL-03 (CMA-03)*
Age/Gender	52/F	72/M	53/M
Disease (status)[#]	PCL (D)	PCL (D)	RRMM
BM infiltration (%PC)	90	60	99
Immunoglobulin type	IgDλ	IgGκ	IgGκ
β_2 microglobulin (mg/mL)	16	14	4
Lytic lesions	Yes	Yes	No
Specimen[¶]	PB	PB	PE

[#]*PCL (D): plasma cell leukemia at diagnosis; RRMM: refractory relapsed multiple myeloma; BM: bone marrow; PC: plasma cells;* [¶]*PB: peripheral blood; PE: peritoneal effusion.*

derived from malignant plasma cells taken during the extramedullary phase of the disease, when such cells are generally characterized by highly proliferative activity. The majority of HMCL are responsive to various cytokines, but they often become independent of specific growth factors (particularly IL-6) because of the selection of subclones characterized by an intracellular autocrine IL-6 loop.[4]

We report here the establishment of three novel HMCL (CMA-01, CMA-02 and CMA-03) derived from peripheral blood or peritoneal effusion plasmablasts of myeloma patients with extramedullary disease and a short survival. The cell lines are IL-6-dependent and therefore represent a suitable model for studies concerning various aspects of the biology of multiple myeloma, particularly the pathways involved in cytokine responses.

Design and Methods

Patients' characteristics

The CMA-01, CMA-02 and CMA-03 HMCL were established *in vitro* from plasmablasts isolated after obtaining informed consent from patients admitted to our Hematology Service: CMA-01 and CMA-02 were derived from peripheral blood plasmablasts of patients with plasma cell leukemia, and CMA-03 from a peritoneal effusion of a patient with refractory myeloma. Table 1 shows the clinical parameters of the patients from whom the plasma cells were isolated.

Establishment and culture of the CMA-01, CMA-02, CMA-03 cell lines

Mononuclear cells (60-90% plasmablasts) from peripheral blood (CMA-01 and CMA-02) or peritoneal effusion (CMA-03) were cultured in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum and 20 U/mL recombinant human IL-6 (R&D System, Minneapolis, MN, USA) at a concentration of 5×10^5/mL in 24-well plates, and incubated at 37°C in a 5% CO_2 humidified atmosphere.

The cells were fed twice weekly by partially replacing the medium and adding fresh IL-6. The cells started growing after 3-6 weeks of culture and they are still maintained under the same culture conditions. Mycoplasma contamination was excluded using the MycoAlert kit (Cambrex Bioscience, Rockland Inc, Rockland, ME, USA). EBV infection was investigated by polymerase chain reaction (PCR) using EBV nuclear antigen-1 specific primers (forward 5'-AAGGAGGGTGGTTTGGAAAG-3'; reverse 5'-AGACAATGGACTCCCTTAGC-3'). The control HMCL, KMS12, was cultured under the same conditions without IL-6.

Immunophenotype analysis

Immunophenotypic characterization of the cell lines was performed using the following panel of monoclonal antibodies: CD45, CD38, CD56, CD126, CD20, CD19 (Becton Dickinson, San Josè, CA, USA) and CD138 (IQ Products, Groningen, The Netherlands). The monoclonal antibodies were used in triple staining, with CD138 being included in all of the combinations in order to identify plasma cells. Irrelevant isotype-matched mouse immunoglobulins were used as negative controls. Fluoroscein isothiocyanate-conjugated rabbit anti-human sera specific for surface or cytoplasmic immunoglobulins were purchased from DAKO (Glostrup, Denmark); fluoroscein isothiocyanate-conjugated rabbit immunoglobulins were used as negative controls. Cell reactivity to the different antibodies was analyzed on a FACSCalibur flow cytometer (Becton Dickinson) using the PAINT-A-GATE-PRO software program (Becton Dickinson). An antigen was considered positive when at least 10% of the plasma cells displayed reactivity for this marker.

Expression analysis of interleukin-6 receptorα (IL-6Rα)

The reverse transcriptase-PCR analysis of IL-6Rα was performed in accordance with a protocol previously described.[6] The protocol used for the quantitative real-time PCR (Q-RT-PCR) has also been published elsewhere.[7] Human *IL6R* and endogenous control *GAPDH* were respectively analysed using assay-on-demand and pre-developed assay reagents (PDAR), (Applied Biosystems, Foster, CA, USA). The number of *IL6R* transcripts in each sample was calculated from a standard curve relative to the U266 cell line, and the results were expressed as the ratio between *IL6R* RNA (ng) and *GAPDH* RNA (ng). All the determinations were made in triplicate. The con-

centration of IL-6Rα in the cell culture supernatants was determined using a sandwich ELISA (R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. The sensitivity of the test was 6.5 pg/mL. The supernatants from the positive (OPM-2) and negative control (Jurkat and SKMM1) cell lines selected on the basis of Q-RT-PCR analysis were also analyzed.

Cytogenetic and fluorescence in situ hybridization (FISH) analyses

The cells were treated with colcemid (0.1 μg/mL) for 90 minutes, harvested using hypotonic potassium chloride, fixed with methanol/glacial acetic acid (3:1 v/v), and stored at –20°C. The chromosome preparations were made according to the conventional method, analyzed by Q-banding, and karyotyped according to the ISCN recommendations (1995). The FISH analyses were performed to investigate the presence of the most frequent translocations found in multiple myeloma, t(11;14)(q13;q32.3), t(4;14) (p16.3; q32.3), t(16;14)(q23;q32.3), t(6;14)(p21.1;q32.3), t(8;14) (q24;q32.3) and t(14;20)(q32;q11), as well as chromosome 13q14 deletion. All of the probes used to detect the chromosomal translocations have been previously described.[8,9] The 13q14 deletions were detected using two BAC clones selected from the UCSC Genome: 320G21 located centromerically to the *RB* gene, and 34F20 located within the minimal deleted region.[10] Preparation of the probes, hybridization of the slides and the FISH analyses were performed as previously described.[8]

Cell growth analysis

The cells were seeded in triplicate at a concentration of 2×10^5/mL in 96-well plates, and counted daily; the medium was partially replaced every other day, and the population doubling time was determined from the growth curve of exponentially proliferating cells. In order to investigate the effect of IL-6 on cell growth, after two washes in medium without IL-6 the cells were seeded in triplicate at a concentration of 2×10^4cells/well in 96-well plates, and different concentrations of IL-6 were added. Cell proliferation was evaluated at different times of culture by means of the Cell Proliferation Reagent WST-1 kit (Roche, Indianapolis, IN, USA) following the manufacturer's instructions. Cloning efficiency was assayed using the soft-agar assay: briefly, the cells were plated in triplicate into 35-mm agar plates containing Iscove's modified Dulbecco's medium, 20% fetal calf serum, 20 U/mL IL-6 and 0.5% purified agar and the colonies were scored after two weeks of incubation at 37°C, 5% CO_2.

DNA content analysis

Cytofluorimetric analysis of DNA distribution of the three exponentially growing myeloma cell lines was performed by means of propidium iodide staining. Briefly, 10^6 cells (previously fixed in 70% ethanol) were incubated in staining solution (propidium iodide 20 μg/mL, RNAse 0.5 mg/mL, Nonidet P-40 0.05%) at room temperature in the dark. The measurements were made after 1 h using a FACSCalibur flow cytometer (Becton Dickinson). The DNA cell cycle analyses were performed using the MODFIT software (Verity Software House, Topshem, ME, USA). The DNA index (DI) was calculated as the ratio of the modal channel of the G_0-G_1 peaks obtained for the cell line and a reference sample (peripheral blood mononuclear cells from a normal donor).

Gene expression profiling analysis

Plasma cells were purified from peripheral blood or peritoneal effusion using CD138 immunomagnetic microbeads as previously described.[11] Total RNA was extracted from the CMA-01, CMA-02 and CMA-03 cell lines, and from purified (>90%) $CD138^+$ tumorplasma cells, using the TRIzol® reagent (InVitrogen, Carlsbad, CA, USA), and biotin-labeled cRNA was generated as previously reported.[11] In accordance with the Affymetrix protocol, 15 μg of fragmented cRNA were hybridized to U133A Probe Arrays (Affymetrix Inc., Santa Clara, CA, USA). After scanning, the images were processed using Affymetrix™ MicroArray Suite (MAS) 5.0 and the gene expression values were determined using the global scaling option, which allows a number of experiments to be normalized to one target intensity (i.e. 100) and thus compared. The data were analyzed using the previously described Genes@Work software, a gene expression analysis tool based on the pattern discovery algorithm Structural Pattern Localization Analysis by Sequential Histograms (SPLASH).[11,12] Briefly, Genes@Work discovers global gene expression *signatures* that are common to an entire set of at least *n* experiments (the support set), where *n* is a user-selectable parameter called the *minimum support*. Differentially expressed genes are identified by comparing an expected gene expression probability density *p(e)* empirically computed from the experimental set with a predefined threshold (*parameter* δ). The patterns of differentially expressed genes are then ranked according to their statistical significance (*z-score*).

Results

The three cell lines were established from bulk cell populations; the time necessary for their establishment was two months for CMA-01 and CMA-03, and one month for CMA-02. The cell lines were characterized at early culture passages.

Table 2. Immunophenotypic, cytogenetic and FISH analysis of the CMA-01, CMA-02 and CMA-03 cell lines.

Characteristic	*CMA-01*	*CMA-02*	*CMA-03*
Cell surface markers (% positive cells)			
CD38 bright	70	80	99
CD138	99	80	88
CD138/45	Neg	55	99
CD138/56	60	80	Neg
CD138/126	63	<10	<10
CD138/20	66	Neg	Neg
CD138/19	59	Neg	Neg
CD138/117	70	Neg	Neg
Surface membrane Ig	IgDλ	IgGκ	IgGκ
Cytoplasm Ig	IgDλ	IgGκ	IgGκ
Cytogenetic analysis	hypotetraploidy	hypotetraploidy	hypotetraploidy
FISH analysis	t(8;14) t(11;14)	t(8;14) t(14;16)	t(8;14) t(20;unknown)

Ig: immunoglobulin.

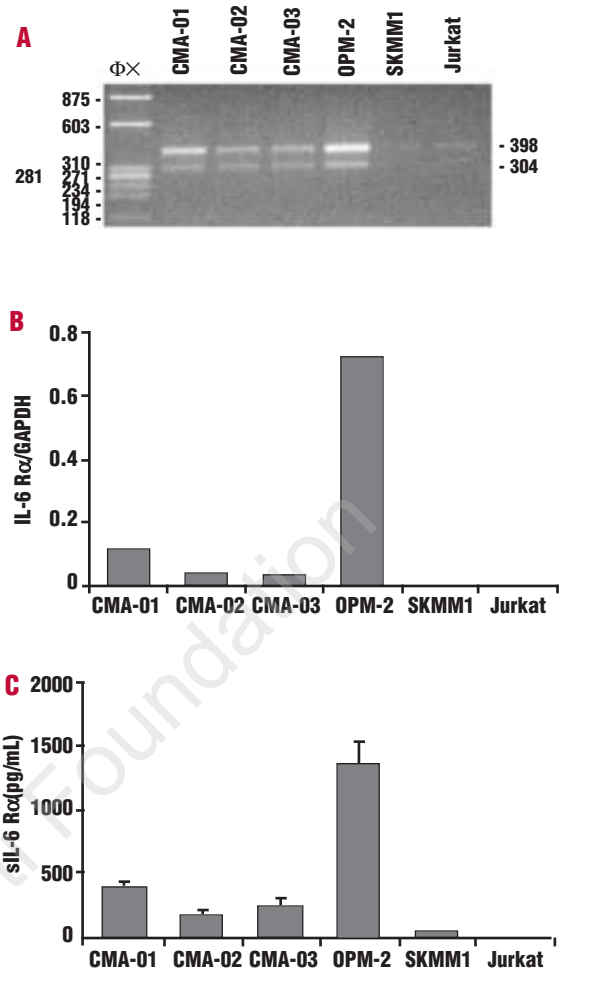

Figure 1. IL-6Rα expression in the CMA-01, CMA-02 and CMA-03 cell lines. (A) reverse transcriptase PCR analysis: the 398 bp and 304 bp fragments corresponding to membrane IL-6Rα and the alternatively spliced IL-6Rα mRNA are shown. (B) Q-RT-PCR analysis of IL-6Rα. (C) sIL-6Rα release by the CMA-01, CMA-02 and CMA-03 cell lines; comparison with OPM2 (positive control) and SKMM1 and Jurkat (negative controls) cells.

Morphological and immunophenotypic features

Morphological analysis of the three cell lines showed large cells with the characteristics of immature plasmablasts with one or more nucleoli. Abnormal plasmablasts with two or three nuclei and mitosis were also observed, particularly in CMA-02 (*data not shown*).

The immunophenotype characterization of the three cell lines is shown in Table 2. The cell lines showed high reactivity for the plasma cell-specific markers (CD38 bright, CD138). CMA-02 and CMA-03 were positive for CD45, an antigen whose expression declines during normal plasma cell differentiation and whose absence is considered to be a poor prognostic factor in multiple myeloma.[13] The CD19 and CD20 B-cell antigens were only expressed on CMA-01 cells: the expression of CD20 (normally absent on plasma cells) may have prognostic value as it is related to advanced stages of the disease and a poor prognosis.[14] All three cell lines expressed the same immunoglobulin type detected in the patients' plasma cells, not only in the cytoplasm but also on the surface membrane; this expression also appears to be related to more aggressive disease.[15] The adhesion molecule CD56, which is frequently expressed in multiple myeloma but not in normal plasma cells and is generally lost during extramedullary disease dissemination,[16] was present in two of the cell lines. CMA-01 expressed CD117 (c-KIT), a class III tyrosine kinase receptor not expressed by normal plasma cells, whose presence in about 30% of multiple myeloma patients makes it a *tumor-associated marker*.[17] Finally, significant levels of CD126, the IL-6 receptor α (IL6-Rα), were detected only in CMA-01, whereas it was almost undetectable (<10% of cells) in CMA-02 and CMA-03. However, appreciable levels of specific IL-6Rα transcripts were found by reverse transcriptase-PCR (Figure 1A) and Q-RT-PCR (Figure 1B). In particular, reverse transcriptase-PCR showed the presence of two transcripts corresponding to the membrane (398 bp fragment) and the alternatively spliced soluble form of IL-6Rα (sIL-6Rα, 304 bp fragment).

To confirm this finding, and test the possibility that the IL-6 signaling could be mediated by the binding of the cytokine to sIL-6Rα, as found in other HMCL and plasma cells from myeloma patients,[18] we used ELISA to determine the release of IL-6Rα in cell supernatants after 48 hours of incubation. As shown in Figure 1C, all of the cell lines produced appreciable levels of sIL-6Rα. As sIL-6Rα acts as an agonist capable of potentiating IL-6 activity, our results suggest that the cytokine signaling is at least partially mediated by this

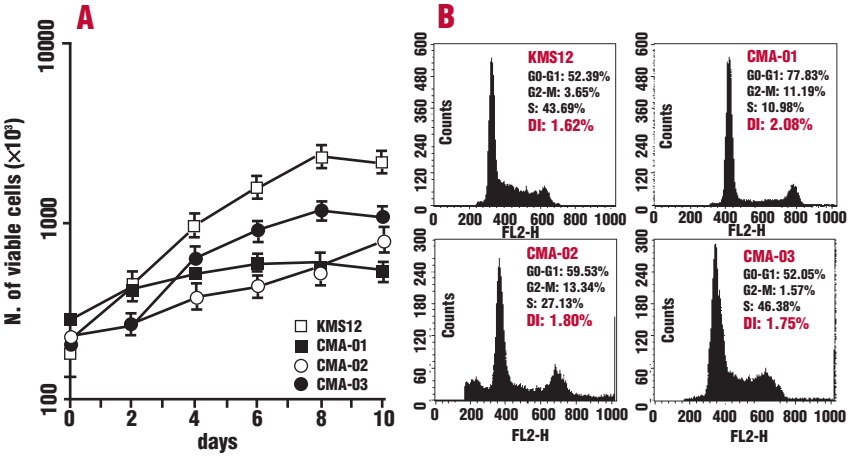

Figure 2. Proliferation characteristics of the CMA-01, CMA-02 and CMA-03 cell lines; the KMS12 cell line was analyzed as a control. (A) Growth curve. (B) Flow cytometric DNA analysis of log phase growing cells. The DNA index (DI) represents the ratio of the modal channel of the G_0-G_1 peak of the cell line and a diploid control sample.

mechanism in our cell lines.

PCR analysis did not detect EBV DNA sequences in any of the cell lines (*data not shown*), thus indicating the absence of EBV-immortalized lymphoblastoid cells in the cultures; this result is particularly relevant in CMA-01 cells, which express CD19.

Cytogenetic and FISH analyses

Conventional cytogenetic analysis of the CMA-01, CMA-02 and CMA-03 cells detected hypotetraploid complex karyotypes and structural alterations (Table 2). The involvement of chromosome 1 in complex rearrangements was observed in all the cell lines,[19] and a t(1;11) translocation was identified in the CMA-03. In addition, we detected the t(11;14) translocation and duplication of the long arm of chromosome 4 in CMA-01, two copies of chromosome $12q^+$ in CMA-02, and one copy of chromosome 9p- in CMA-03. The karyotypes of CMA-02 and CMA-03 were comparable with those of the primary tumor cells, confirming the fidelity of the cell lines with the original samples. Unfortunately, the karyotype of the original plasma cells from which CMA-01 was derived was not available. In order to characterize the chromosome alterations in these cell lines better, we used FISH analyses to evaluate the presence of the main translocations identified so far in multiple myeloma, and the evidence of the chromosome 13q14 deletion. As shown in Table 2, all three cell lines were positive for the t(8;14) translocation involving the *MYC* locus which, in all cases, was the result of a complex chromosomal rearrangement with different extra *MYC* and *IGH* signals (*data not shown*). The classical t(11;14) translocation involving the *CCND1* locus was found in CMA-01, thus confirming the cytogenetic results. We also observed two extra *CCND1* locus signals on an apparently normal chromosome 11 and an unidentified marker. CMA-02 showed a t(14;16) translocation involving the *MAF* locus, and four different co-localized signals were detected on two putative der(14) and two unidentified chromosomes. Furthermore, we detected various extra-signals of probes specific for the *MAF* or *IGH* locus (*data not shown*). The CMA-03 cell line showed a complex pattern of signals involving the *MAFB* locus on two putative chromosomes 20, two der(20) and an unidentified marker without the involvement of any immunoglobulin loci. Finally, none of the cell lines showed the 13q14 deletion, while the detection of 3-4 copies of chromosome 13 was in line with their hypotetraploid status. These findings confirmed the presence of many numerical and structural chromosomal abnormalities in the HMCL, which thus reflect the high genomic instability characteristic of multiple myeloma.

Growth characteristics

The proliferation patterns of the three cell lines were characterized by analyzing their growth curves, IL-6 dependency and cloning efficiency. The cell lines grow in suspension as single cells or small clusters. As shown in Figure 2A, the doubling times of CMA-01, CMA-02 and CMA-03 were respectively 90, 110 and 80 hours (slower than that of the control KMS12 cells). As frequently found in recently established HMCL, the cells reach a relative low saturation cell density (8×10^5/mL), while KMS12 control cells grow to high saturation density (2×10^6 cells/mL). Cytofluorimetric analysis of DNA distribution in exponentially proliferating cells confirmed the proliferating pattern of the cell lines (Figure 2B). All three cell lines grow in soft agar, albeit with a low level of cloning efficiency (0.5-1%) (*data not shown*). In order to confirm the IL-6 dependency of the three cell lines after their establishment in culture, we analyzed the metabolic activity of viable cells in the absence or presence of different IL-6 concentrations. As shown in Figures 3A and 3B, the cytokine is essential for CMA-01, CMA-02 and CMA-03 proliferation: CMA-03 cannot grow without the supplementation of IL-6, and the rate of growth of CMA-01 and CMA-02 is very slow in its absence. This may be due to the presence of cell sub-clones that do not depend on IL-6 pro-

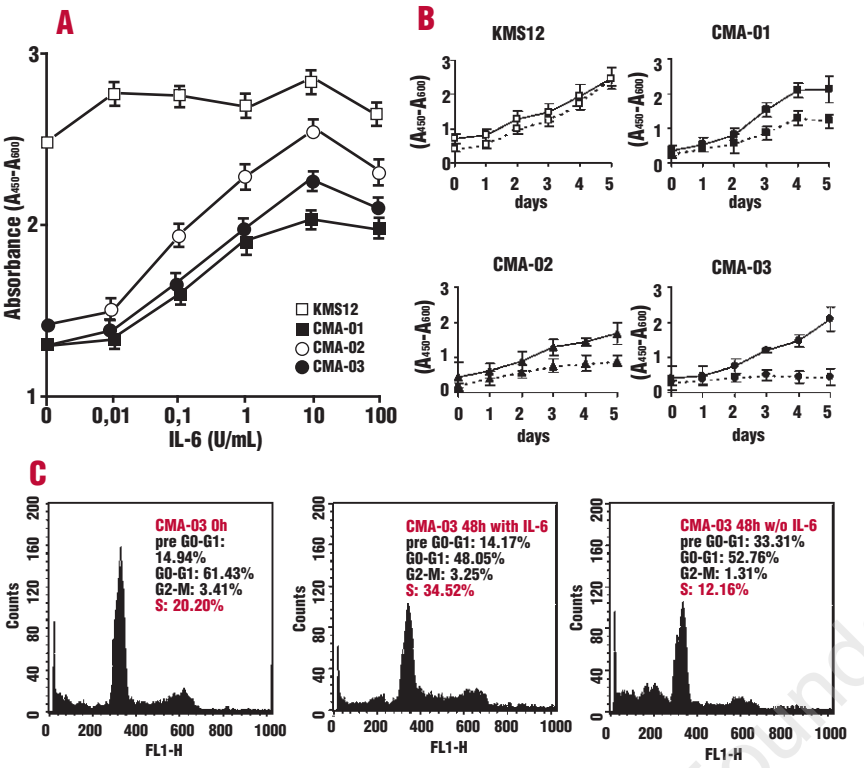

Figure 3. Effect of exogenous IL-6 on the proliferation of the CMA-01, CMA-02 and CMA-03 cell lines. (A) Increasing amounts of recombinant human IL-6 were added to the medium, and cell proliferation was evaluated after 72 hours by measuring the metabolic activity of viable cells. (B) Proliferation of cells lines in the presence (black lines) or absence (dotted lines) of IL-6 (20 U/mL). (C) Flow cytometric cell cycle analysis by DNA content in the CMA-03 cell line in the presence or absence of IL-6 (20U/mL).

liferating activity but, as we failed to isolate them by growing the cells without IL-6 for 2-3 weeks, it is probable that they are poorly represented in our cell populations or that IL-6 requirement is only partially overcome. In order to investigate whether IL-6 acts on the proliferation rate and/or as a survival factor, we analyzed cell cycle status by evaluating DNA content in the CMA-03 cell line in the presence or absence of the growth factor. As shown in Figure 3C, a proportion of cells in the S phase was found in the presence of IL-6 after 48 hours, thus suggesting that the cytokine promotes cell proliferation. The moderate increase of pre-G_0-G_1 rate in the absence of IL-6 is possibly related to the presence of apoptotic cells, thus suggesting that in our cell lines IL-6 may also increase cell survival upon specific stimuli.

Gene expression profiling

To identify the gene expression pattern(s) possibly related to the *in vitro* growth of multiple myeloma plasmablasts, we used supervised analysis to compare the global gene expression profiles of the cell lines with those of purified tumor plasmablasts. The analysis selected a number of transcripts as differentially expressed in the cell lines versus primary tumor plasma cells (16 up- and 46 down-regulated; Figure 4). Several proliferation-associated genes were up-regulated in the CMA samples, including genes controlling DNA metabolism such as *DNAse 2*, *RRM2*, *ZWINT* and *TYMS*. Interestingly, the most up-regulated gene in the CMA cell lines was *ADAM22*, a member of the disintegrin-metalloproteinase protein family that modulates cell-cell and cell-matrix interactions and protease activity, whose expression is enhanced in cancer cells and metastases;[20] it is also worth noting the higher expression of the CD71 transferrin receptor in the CMA cell lines, a common characteristic of all HMCL.[4] In contrast, the majority of down-regulated genes in the CMA cell lines are associated with anti-proliferative or pro-apoptotic functions (e.g. *ISG20*, *FCRH2* and *KLF2*). Notably, we also found specific down-regulation of the gp130 catalytic subunit of IL6-R (*IL6ST*), a transducer chain shared by many cytokines and growth factors.[21] Genes codifying for molecules involved in cell adhesion pathway, such as chemokine receptors (*CCR2*, *CXCR4*) and protein kinase Cβ (*PRKCB1*), whose signal transduction pathway triggers the effects of vascular endothelial growth factor on plasma cell migration, were also specifically down-regulated in the CMA samples. Unexpectedly, we found down-regulation of the genes encoding *ANXA2* (involved in plasminogen-mediated cell invasiveness) and its ligand *S100A10*, both of which are over-expressed in cancer cells and particularly in some cases of multiple myeloma and HMCL.[22] Overall, these results show that cell line establishment involves the clonal selection of rapidly proliferating cells that have probably acquired further independence from microenvironment homing factors that are particularly relevant to myeloma biology.

The availability of global gene expression profiles makes it possible to investigate the expression levels of selected genes of particular interest. In line with the

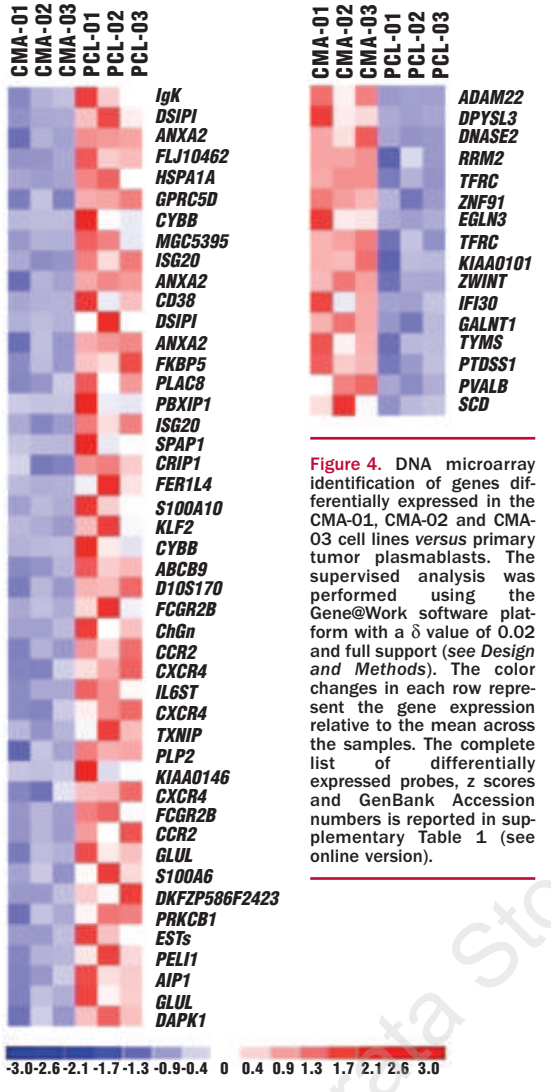

Figure 4. DNA microarray identification of genes differentially expressed in the CMA-01, CMA-02 and CMA-03 cell lines *versus* primary tumor plasmablasts. The supervised analysis was performed using the Gene@Work software platform with a δ value of 0.02 and full support (see *Design and Methods*). The color changes in each row represent the gene expression relative to the mean across the samples. The complete list of differentially expressed probes, z scores and GenBank Accession numbers is reported in supplementary Table 1 (see online version).

results of FISH (Table 2) and Q-RT-PCR (*data not shown*), the analysis of the genes targeted by the main chromosomal translocations found in multiple myeloma showed that CMA-01 was characterized by deregulated expression of *CCND1*, CMA-02 by *MAF*, and CMA-03 by *MAFB*, while all of the cell lines were positive for expression of the *MYC* and *WHSC1* genes (Figure 5A). In particular, the expression of *WHSC1*, even though at lower levels than those found in t(4;14) samples, is consistent with previous data indicating the expression of this gene in plasma cell leukemia plasma cells negative for t(4;14).[11] Given recent data supporting the important role of cyclin D deregulation in multiple myeloma,[23] we also analyzed the expression levels of cyclin D genes (Figure 5A). The results showed the presence of deregulated expression of at least one of them in each of the HMCL, in agreement with the gene expression data deriving from the analysis of the patients.

In order to characterize the cell lines more extensively, we analyzed the expression profiles of genes encoding proteins known to be involved in myeloma biology (Figures 5B-5C). Figure 5B shows the expression profiles of genes coding for molecules involved in the cytokine pathway. No *IL-6* gene expression was detected in any of the three cell lines, whereas *CD126* (IL-6Rα) was expressed in all of the samples, albeit at low levels, indicating the persistence of the IL-6-dependence. Interestingly, both *IGF1* (known to be a potent survival and proliferation factor for multiple myeloma cells) and its receptor were moderately expressed, while *VEGF* was expressed in all the samples. No expression could be detected for the mRNA of other proteins known to be involved in cytokine pathways, such as IL1-β, IL-7, IL-10, IL-11 or IL-21. Among the

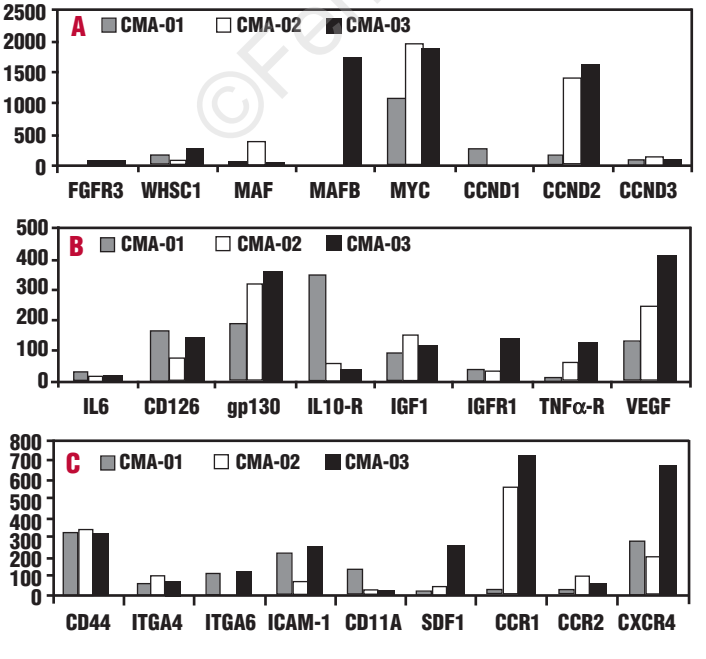

Figure 5. mRNA expression profiles of selected transcripts in CMA-01, CMA-02 and CMA-03 cell lines as assessed by DNA microarray analysis. The values on the vertical axis represent the fluorescence intensity of streptavidin-PE-stained biotinylated cRNA hybridized to a specific probe set on HG-U133A arrays. For the complete list of Affymetrix probe set ID, see Supplementary Table 2. (A) Gene targets of *IGH* translocations and cyclin D genes; (B) genes involved in cytokine pathways; (C) genes involved in cell adhesion pathways.

transcripts specific for proteins involved in the cell adhesion pathways (Figure 5C), only a few were expressed at high level, including CD44, previously described to be highly expressed in the majority of HMCL,[4] and the chemokine receptor CCR1. In addition to this receptor, *CXCR4* was also expressed in all of the cell lines, although at lower levels than those in the primary tumor samples, as shown by the supervised analysis (Figure 4).

Discussion

Few IL-6-dependent HMCL are currently available, as long-term cultivation selects cells that develop an autocrine IL-6 loop with an increased expression of IL-6 and IL-6R. Although the establishment of a cell line is *per se* a clonal or oligoclonal selection of the original population, HMCL that retain their growth dependence on IL-6 may be useful in specific studies, such as when investigating the signaling cascade mediating the growth, survival and cell cycle regulatory effects triggered by IL-6, or identifying the pathways that may make multiple myeloma cells independent of the growth factor. To this end, the characterization of global transcriptional profiles may be a useful tool. Indeed, we are now attempting to select IL-6-independent clones in the CMA-01 and CMA-02 cell lines after several passages in culture in order to investigate the biological pathways involved in this process.

DV, cell culture experiments and biological analyses; MM, gene expression profiling analysis and editing of the manuscript; SF, FISH experiments; LN, immunophenotype analyses; DI, molecular characterization SG, cytogenetic analyses; KT, cell culture experiments; AZ and GLD, critical revision of the paper and final approval; LL, conception and design of the study, interpretation of results, drafting and critical revision of the manuscript; AN, critical revision of the paper and final approval. All authors approved the final version of the article to be published. The authors declare that they have no potential conflicts of interest.

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