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Abstract

A plethora of myeloma growth factors (MGF) has been identified, but their relative importance and cooperation has not been determined. We investigated 5 well-documented MGF (IL-6, IGF-1, HGF, HB-EGF, APRIL) in serum-free cultures of human myeloma cell lines (HMCLs). In all of 3 $CD45^-$ HMCLs, an autocrine IGF-1 loop promoted autonomous survival. To the contrary, all 5 $CD45^+$ HMCLs could not survive and required addition of either IL-6 (5/5), IGF-1 (4/5), HGF (1/5), HB-EGF (1/5) or APRIL (1/5). IGF-1 was the major MGF since its activity was abrogated by an IGF-1R inhibitor only, whereas IL-6, HGF or HB-EGF activity was inhibited by both IGF-1R- and receptor-specific inhibition. APRIL activity was inhibited by its specific inhibitor only.

Of the investigated MGF and their receptors, only expressions of *IGF-1R* and *IL-6R* in myeloma cells (MMC) of patients delineate a group with adverse prognosis. This is mainly explained by a strong association of *IGF-1R* and *IL-6R* expression and t(4;14) translocation, but *IGF-1R* expression in MMC, without t(4;14) can also have a poor prognosis. Thus, IGF-1 targeted therapy – eventually in combination with anti-IL-6 therapy - could be promising in a subset of patients with MMC expressing IGF-1R.

Introduction

Multiple myeloma (MM) is a clonal plasma-cell disorder. Multiple myeloma cells (MMC) from almost all patients harbor chromosomal abnormalities by FISH [1,2] and aberrant gene expression [3] at diagnosis in symptomatic disease. These abnormalities are not sufficient to promote MMC growth ex vivo and the tumor microenvironment expresses adhesion molecules and produces myeloma growth factors (MGF) that are critical to trigger MMC survival [4,5]. A plethora of MGF have been identified: interleukin-6 (IL-6) [6], soluble IL-6 receptor [7], the IL-6 family [8], insulin-like growth factor type 1 (IGF-1) [9,10], BAFF/APRIL B cell growth factors [11,12], the epidermal growth factor (EGF) family [13], hepatocyte growth factor (HGF) [14], tumor necrosis factor (TNF) [15], the Wnt family [16], IL-10 [17], IL-21 [18], and the NOTCH ligand family [19]. Some MGF can be produced by the tumor environment (IL-6, BAFF/APRIL, IGF-1, EGF family, Wnt family, HGF) or by MMC themselves (IL-6, IGF-1, HGF, EGF family, Wnt family, Notch ligand family) [20,21]. These MGF activate their specific receptors that in turn result in the activation of several signal transduction pathways [22] including the Janus Kinase/signal transducer and activator of transcription 3 (JAK/STAT3), PI-3 kinase/AKT, Ras/mitogen-activated protein kinase (MAPK), Nuclear Factor-kappa B (NF-κB), and the β-catenin pathway. In a minority of patients with extramedullary proliferation, MMC may grow without the support of the bone marrow microenvironment and human myeloma cell lines (HMCLs) can be obtained. The numerous MGF make it difficult to understand their respective role in the natural history of the disease and whether they are necessary and sufficient or redundant. This is especially relevant since MGF can cooperate to enhance MMC growth as for IL-6 and EGF family members [23], IL-6 and FGF [24], and IL-6 and IGF-1 [25]. This is complicated by the fact that some growth factors are autocrinely produced or present

in the culture medium, thus masking their contribution. Regarding IL-6 and IGF-1, various data were reported using different techniques and focussing on different aspects, which could yield to challenging conclusions. Descamps *et al.* have reported that IGF-1 MGF activity was restricted to $CD45^-$ HMCLs, unlike the $CD45^+$ HMCLs [26]. IL-6 could efficiently trigger the growth of $CD45^+$ HMCLs and the IL-6 activity was unaffected by an IGF-1 inhibitor. An explanation was that the phosphastase activity of CD45 downregulates the kinase activity of IGF-1R, making the $CD45^+$ HMCLs unsensitive to IGF-1. Abroun *et al.* have shown that IL-6 can trigger membrane IL-6R binding to IGF-1R and induce IGF-1R phosphorylation independently of addition of IGF-1 [25]. In this study, IL-6 is a major MGF making it possible to trigger both gp130 and IGF-1R phosphorylation in case of high IL-6R expression. Mitsiades *et al.* have shown that the importance of serum IGF-1 to support the IL-6 dependent growth of the ANBL6 cell line [10]. Regarding the other MGF, in particular HGF, EGF family or BAFF/APRIL, their respective role was not studied comparatively yet.

Another major question is the in vivo relevance of these MGF. Serum levels of IL-6 and soluble IL-6R and of IGF-1 were linked with bad prognosis [27,28]. Divergent data exist regarding the prognostic value of IGF-1R on MMC. With a small cohort of 37 newly-diagnosed patients, Bataille *et al.* have shown that IGF-1R expression on MMC, detected by FACS analysis, had poor prognosis value [29]. Using a cohort of 72 newly-diagnosed patients and *IGF-1R* expression detected by Affymetrix microarray, Chng *et al.* [30] failed to find a prognosis value of *IGF-1R* expression, whereas *IGF-1R* expression was increased in poor prognosis groups. The prognostic value of the other MGF receptors was not documented yet.

To look for a possible ranking of five well-documented MGF, we used a defined serum-free culture medium able to sustain growth of all our HMCLs to avoid

unidentified components present in serum, in particular IGF-1, which might confound interpretation of the results. We also look for the prognostic value of MGF receptor gene expression on MMC using two independent large patient cohorts.

Materials and Methods

Cell samples

The 9 human myeloma cell lines (HMCLs) were obtained in our laboratory [31] or purchased from ATCC (Rockville, MD, USA). They were maintained in RPMI1640 (Gibco Invitrogen, France), 10% fetal bovine serum (FBS, PAA laboratory GmbH, Austria) and for the IL-6-dependant cell lines, with 2 ng/ml of IL-6 (Abcys SA, Paris, France). Normal bone marrow plasma cells (BMPC) were obtained from healthy donors after informed consent was given. Plasma cells, $CD27^{+}$ memory B cells (MBC) and polyclonal plasmablasts (PPC, $CD38^{++}$, $CD20^{-}$) were obtained as previously described [32].

MMC of 171 patients with previously-untreated MM were included after written informed consent was given at the University hospitals of Heidelberg (Germany) or Montpellier (France). These 171 patients were treated with high dose therapy (HDC) and autologous stem cell transplantation (ASCT) and were termed in the following Heidelberg-Montpellier (HM) series. 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 [33] and termed in the following LR-TT2 series. These data are publicly available (GEO, http://www.ncbi.nlm.nih.gov/geo/, accession number GSE2658).

Reagents

Human recombinant (r) IL-6 (rIL-6), rIGF-1 and rHGF were purchased from Abcys SA (Paris, France), human rHB-EGF, rAPRIL, anti-human HGF monoclonal antibody (MoAb) and BCMA-Fc from R&D Systems (Minneapolis, MN). The B-E8 anti-IL6 MoAb was a generous gift from Dr Wijdenes (Diaclone, Besancon, France) [34], the NVP-AEW541 IGF-1R inhibitor from Novartis Pharma AG (Basel, Switzerland) [35] and

PD169540 pan-ErbB kinase inhibitor from Pfizer Global Research and development (Ann Arbor, MI, USA). We used Syn H, an Iscove-based fully-defined culture medium containing human albumin without insulin (ABCell-Bio, Montpellier, France).

Interphase FISH, microarray hybridization, Real-time RT-PCR

Interphase-FISH-analysis was performed according to our previously reported standard protocol [2]. RNA was extracted and hybridized to human Affymetrix microarrays as previously described [36]. *IGF-1R* expression was evaluated by real-time RT-PCR using the assays-on-demand primers and probes and the *Taq*Man Universal Master Mix (Applied Biosystems, Courtaboeuf, France) as reported [36].

Flow cytometry Analysis

The expression of CD45 isoforms and IGF-1R on HMCLs were evaluated by incubating 5 x 10^5 cells with PE-conjugated anti-CD45RO, anti-CD45RA (Immunotech, Marseille, France), anti-CD45RB (BD Biosciences, San Diego, CA), anti-IGF-1R (Santa Cruz Biotechnology, Santa Cruz, CA) or an isotype-matched control antibody in phosphate-buffered saline (PBS) and flow cytometry analysis was performed on a FACScan (Becton Dickinson, San Jose, CA).

Growth assay for myeloma cells

HMCLs were IL-6- and serum-starved for 2 hours and cultured for 4 days in 96-well flat-bottomed microtiter plates in serum-free culture medium without cytokine (control) or with either rIL-6 (200 pg/mL), rIGF-1 (100 ng/mL), rHB-EGF (1 µg/mL), rHGF (200 ng/mL), or rAPRIL (200 ng/mL), without or with the B-E8 anti-IL-6 MoAb (10 µg/mL), the IGF-1R inhibitor NVP-AEW541 (1 µM), the anti-HGF MoAb (25 µg/mL), the PD169540 pan-ErbB kinase inhibitor (1 µM), or BCMA-Fc (10 µg/mL). In some experiments, myeloma cells were grown with graded IGF-1 concentrations. The growth of myeloma cells was evaluated by quantifying intracellular ATP amount with a

Cell Titer Glo Luminescent Assay (Promega Corporation, Madison, USA) with a Centro LB 960 luminometer (Berthold Technology, Germany).

Signal transduction, IGF-1 production and immunoblot analysis.

To look for signal transduction, myeloma cell lines were starved for 18 hours, washed and then incubated with the various pre-warmed MGF with or without inhibitors for 20 minutes. Cells were lysed and transferred to a nitrocellulose membrane (Schleicher and Schuell, Kassel, Germany), as previously described [36]. Membranes were immunoblotted with a rabbit anti-IGF-1 (Abcam, Cambridge, United Kingdom), anti-phospho-Akt, anti-phospho-MAPK, anti-phospho-Stat3, anti-Akt, anti-MAPK antibodies (Cell Signaling Technology, Beverly, MA) and with a mouse anti-Stat3 antibody (Cell Signaling technology). As a control for protein loading, we used a mouse monoclonal anti-β-actin antibody (Sigma, St Louis, MO). The primary antibodies were visualized with goat anti-rabbit (Sigma) or goat anti-mouse (Bio-Rad, Hercules, CA) peroxidase-conjugated antibodies by an enhanced chemiluminescence detection system.

Measurement of cytokine concentration by ELISA.

HMCLs were cultured for 2 days in Syn H serum-free culture medium without cytokine and IL-6 and IGF-1 in the culture supernatant were measured using ELISA kits with a detection level of 3 pg/ml and 45 pg/ml respectively (R&D, Minneapolis, MN).

Spiked *MMSET* expression surrogating t(4;14).

The t(4;14) translocation results in aberrant *FGFR3* expression in 70% of patients and *MMSET* spiked expression in 100% of patients [37] and spiked *MMSET* expression has been taken as surrogate for the presence of t(4;14) [3]. In our series of 94 patients with FISH analysis, 20/94 patients had t(4;14) resulting in aberrant *FGFR3*

expression in 16/20 and spiked *MMSET* expression (range of Affymetrix signal 500-2500) in 19/20 using the 222777_s_at *MMSET* probe set with the highest variation coefficient among MMC samples. In the 74 patients lacking t(4;14), no *FGFR3* and a low *MMSET* expression (Affymetrix signal: 1-300) was found. We defined a spiked *MMSET* gene if *MMSET* signal ≥ Q3 + 3 (Q3-Q1) with Q3 and Q1 being the *MMSET* signals of the first and third quartile of MMC samples. Using this definition, 19/20 (95%) of patients with t(4;14) had spiked *MMSET* and only 2/74 lacking documented t(4;14) had spiked *MMSET*.

Statistical analysis

A difference in the mean values of two (paired) groups was evaluated with a (paired) student *t* test using the SPSS10 software. Gene Expression Profiles were analyzed with our bioinformatics platform (RAGE, http://rage.montp.inserm.fr) [38] and with the Amazonia website [39]. The prognostic value of a probe set was evaluated combining Affymetrix data obtained with human genome U133 set or U133 Plus 2.0 microarrays. We used the Affymetrix call ("present" or "absent") that is determined by the Affymetrix GCOS-software as indicator whether a gene is expressed or not. When a probe set was absent in MMC of a fraction of patients (*IGF-1R* and *c-Met*), the survival of patients with MMC with a present or absent call was compared. When a probe set was present in MMC of all patients (*IL-6R*, *gp130*, *TACI*, *BCMA*), the survival of patients with a signal below or above the median signal was compared. The statistical significance of differences in survival between groups of patients was calculated by the log-rank test. An event was defined as relapse or death (for EFS) or as death (for OAS). Multivariate analysis was performed using the Cox proportional hazards model. Survival curves were plotted using the Kaplan-Meier method.

Results

Autocrine IGF-1 is a critical survival factor of autonomously-surviving CD45$^-$ HMCLs in serum-free culture medium.

Without adding exogenous MGF and in serum-free culture medium, the number of viable cells in 5 HMCLs - XG-1, XG-2, XG-6, XG-12, XG-14 - was decreased at day 4 of culture (Figure 1A) ($P \leq .05$). This effect was observed with cell concentrations ranging from 4 x 10^3 to 4 x 10^5 cells/mL. The survival of 3 HMCLs – XG-5, XG-20, XG-7 - was not affected, and XG-7 showed even an increased growth ($P \leq .05$) (Figure 1A). A difference between autonomously and non-autonomously-surviving HMCL is CD45 expression. The 3 autonomously-surviving HMCLs expressed the 3 CD45 isoforms either at a low level or not at all (≤ 6%, Figure 1B), and the 5 non-autonomously-surviving HMCLs expressed at least two isoforms, mainly CD45RO and CD45RB. CD45RA$^+$ HMCLs are less frequent and their behavior may not always be representative of the wider spectrum of HMCLs (Figure 1B). Looking for the simultaneous expression of genes coding for a growth factor and its receptors using Affymetrix microarrays, a possible autocrine IL-6/IL-6R/gp130 loop is found in 6/8 HMCLs, an IGF-1/IGF-1R loop in 7/8, an HGF/c-met loop in 2/8, BAFF-APRIL/BAFFR-BCMA-TACI loops in 3/8, and EGF family/ErbB family loops in 8/8 (results not shown). To investigate *IGF-1R* expression, we used the 203627_at probe set, which is the only one of 8 *IGF-1R* probe sets that correlated with *IGF-1R* expression assayed by RT-PCR (r = .85, P = .002) and FACS analysis using HMCLs (r = .67, P = .03).

The survival of the 3 autonomously-surviving CD45$^-$ HMCLs was strongly inhibited (50 to 95%, $P \leq .05$) by the NVP-AEW541 IGF-1R kinase inhibitor (Figure 1C). The ErbB inhibitor partially affected XG-7 survival (40% reduction, $P \leq .05$). The inhibitors

to IL-6, HGF, or BAFF/APRIL did not affect these 3 HMCLs. The specificity of NVP-AEW541 IGF-1R kinase [35] has been confirmed in our hands, *i.e.* by the lack of inhibition of the IGF-1R$^-$ XG-12 HMCL (see below), the inhibition of the transduction pathways activated by IGF-1 and the lack of inhibition of those induced by IL-6 and HGF (Figure 2A). We also looked for the production of autocrine IGF-1 or IL-6. IL-6 could be detected in the culture supernatants of some cell lines (XG-1, XG-2 and XG-6) in agreement with previous reports [40]. IGF-1 protein was detected by western blot in the HMCLs that expressed *IGF-1* gene by Affymetrix microarrays (Figure 2B), but IGF-1 concentration in HMCL culture supernatants was below Elisa detection limit (≥ 45 pg/ml).

IL-6 and IGF-1 are the major MGF and rIL-6-, HGF- and HB-EGF-mediated survival of HMCLs is dependant on the presence of an autocrine IGF-1 loop whereas the activity of recombinant APRIL is not.

rIL-6 significantly stimulated ($P \leq .05$) the growth of 2/3 CD45$^-$ HMCL and of 5/5 CD45$^+$ HMCL and IGF-1 of 3/3 CD45$^-$ and 4/5 CD45$^+$ HMCLs (Figure 3). The CD45$^+$ IGF-1R$^-$ XG-12 HMCL was not stimulated by rIGF-1. The lowest concentration of rIGF-1 stimulating these HMCLs was 27 pg/mL (Figure 2C) - below ELISA detection limit (≤ 45 pg/mL) - and that of rIL-6 was 2 pg/mL [40]. The median rate of stimulation by IL-6 and IGF-1 was 2.2 and 2.8 fold for the CD45$^-$ HMCLs ($P \leq .05$) and 9.9 and 5.3 fold for the CD45$^+$ HMCLs ($P \leq .05$) (Figure 3), respectively. The other 3 MGF stimulated significantly 1 or 2 of the 8 HMCLs (Figure 3). These data indicated that, of the factors investigated, IL-6 and IGF-1 are the most important MGF. APRIL, HB-EGF or HGF stimulated only 1 or 2 of the 8 HMCLs. Regarding transduction pathways, IGF-1 and HGF activated AKT and MAPK phosphorylations, unlike STAT3, IL-6 induced STAT3 and MAPK phosphorylations, unlike AKT and their

activity was blocked by their specific inhibitors unlike other MGF inhibitors (Figure 2A). The NF-κB pathway (p65 phosphorylation) could not be switched off by MGF starvation in the investigated HMCLs, suggesting a constitutive activation (results not shown).

In order to investigate a possible cooperation of exogenous and autocrinely active MGF, the 8 HMCLs were coincubated with each of the 5 recombinant MGF and each of the inhibitors of the 5 MGF. Detailed results for 5 HMCLs, XG-1, XG-2, XG-5, XG-7, XG-20, are shown in Figure 4A and data for the 8 HMCLs summed up in Figure 4B. The IGF-1-induced stimulation of the 7 IGF-1-sensitive HMCLs was inhibited by IGF-1R inhibitor only (median inhibition 100%, range 88-100%, $P \leq .05$). The IL-6-induced stimulation of the 7 IL-6-sensitive HMCLs was inhibited by the B-E8 anti-IL-6 MoAb (median inhibition 100%, range 91-100%, $P \leq .05$). IL-6-induced stimulation was also inhibited by IGF-1R inhibitor for 6/7 HMCLs (median inhibition 73%, range 31-99%, $P \leq .05$), unlike the XG-12 HMCL that did not express IGF-1R (Figure 4B). It was unaffected by the other 3 inhibitors (pan-ErbB kinase inhibitor, anti-HGF MoAb and BCMA-Fc BAFF/APRIL inhibitor). Two HMCLs are stimulated by HGF – XG-2 and XG-7 – and the HGF effect was blocked by the anti-HGF MoAb and the IGF-1R inhibitor (Figure 4A, $P \leq .05$) and unaffected by IL-6, ErbB and BAFF/APRIL inhibitors. The same is true for HB-EGF stimulation in XG-2 cells. It is inhibited by the ErbB kinase inhibitor and by IGF-1R inhibitor also. The XG-1 HMCL was stimulated by APRIL and this effect was blocked by the BCMA-Fc APRIL inhibitor but not influenced by the other 4 inhibitors (Figure 4A, $P \leq .05$).

Expression of MGF-receptors

The expression of 6 genes coding for receptor complexes of 4 of the 5 MGF – *IGF-1R, IL-6R, gp130, c-Met, TACI, BCMA* – could be evaluated with Affymetrix U133 2.0

Plus microarrays. *IL-6R, gp130 and BCMA* expressions are up-regulated throughout B cell to plasma cell differentiation ($P \leq .05$), unlike *TACI. IGF-1R* is not expressed in MBC, PPC, BMPC, but expressed in MMC in a fraction of the 123 patients (Figure 5).

Prognostic value of MGF-receptor expression

Among the 6 receptors, only *IGF-1R* (probe set 203627_at) and *IL-6R* (probe set 205945_at) expression had prognostic value in the 2 independent series of newly-diagnosed patients, the HM series of 171 patients and LR-TT2 series of 345 patients. The *IGF-1R* probe set had a present call in the MMC of 31% and 50% of patients in the HM and LR-TT2 series, respectively. *Gp130*, *BCMA* and *TACI* were present in all MMC samples and *c-Met* in MMC of 56% and 39% of patients of the 2 series, respectively. Patients with *IGF-1R*^{absent} MMC had a longer median event-free survival than patients with *IGF-1R*^{present} MMC (P = .006 and .005, Figures 6A and 6B). The same holds true for patients with *IL-6R*^{low} MMC and *IL-6R*^{high} MMC (P = .006 and .004, Figures 6E and 6F). The median EFS of the whole cohort in the 2 series were 1077 and 1604 days respectively. Patients with *IGF-1R*^{absent} MMC had a longer OAS than patients with *IGF-1R*^{present} in the 2 patient series (P = .02 and 3.10^{-4}, Figures 6C and 6D). Patients with *IL-6R*^{low} MMC had also a longer OAS than patients with *IL-6R*^{high} MMC (P = .005 and .008, Figures 6G and 6H).

We found a link between clinical data and presence or absence of *IGF-1R* in MMC: IgA subtype and serum level of lactate dehydrogenase and between *IL-6R*^{high} and *IL-6R*^{low} MMC (IgA subtype) ($P \leq .05$, Table 1). Of note, the frequency of patients with high LDH levels (an adverse prognostic factor) is increased in patients with *IGF-1R*^{absent} MMC (with a better prognosis). Others clinical data - age, light or heavy chain isotype, occurrence of bone lesions, serum levels of β2-microglobulin, albumin, hemoglobin, C-reactive protein or ISS stage - were not significantly different between

IGF-1R^{present} and *IGF-1R*^{absent} or *IL-6R*^{high} and *IL-6R*^{low} groups (Table 1). Genetic abnormalities were assayed in 79 to 129 (depending on the abnormality) patients of the 171 HM patients series (Table 2). Patient groups with MMC showing a presence of t(4;14), 1q21 or del13 had an significantly increased frequency of *IGF-1R*^{present} MMC or *IL-6R*^{high} MMC, respectively, with del17 an increased frequency of patients with *IGF-1R*^{present} MMC, with t(11;14) a decreased frequency of patients with *IL-6R*^{high} MMC ($P \leq .05$) (Table 2). Thus a possible explanation for the adverse prognosis of *IGF-1R*^{present} MMC or *IL-6R*^{high} MMC groups is the high fraction of patients with the poor prognosis t(4;14) translocation in these groups. As documented t(4;14) was not available for all our HM patients and for LR-TT2 patients, we used spiked *MMSET* expression to further analysis a link between spiked *MMSET* and *IGF-1R*^{present} MMC or *IL-6R*^{high} MMC. 15% (26/171) and 14% (49/345) of patients had spiked *MMSET* in HM and LR-TT2 series respectively. Among the patients with spiked *MMSET*, 77% and 73% had also spiked *FGFR3* expression in agreement with reported data [1,3,37]. In the HM and LR-TT2 series, patients with spiked *MMSET* have an increased frequency of patients with *IGF-1R*^{present} MMC compared to patients with unspiked MMSET (77% vs. 23%, $P = 2.10^{-14}$ for HM series and 94% vs. 43%, $P = 8.10^{-15}$ for LR-TT2 series) or with *IL-6R*^{high} MMC (77% vs. 45% $P = 6.10^{-4}$ for HM series and 82% vs. 45% $P = 5.10^{-8}$ for LR-TT2 series). Considering the 8 patients subgroups defined by Zhan *et al*. using GEP [3], the frequency of patients with *IGF-1R*^{present} MMC is increased in PR and MS groups and decreased in HY and CD1 groups ($P \leq .05$). The frequency of patients with *IL-6R*^{high} MMC is increased in PR, LB, MS, and MF groups and decreased in CD1 and CD2 groups ($P \leq .05$) (Table 3). Of note, patients with *IGF-1R*^{present} MMC and lacking spiked *MMSET* had decreased OAS compared to patients with *IGF-1R*^{absent} MMC in the HM and LR-TT2 series, but increased EFS (in

the 2 series) and OAS (in the LR-TT2 series) compared to patients with spiked *MMSET* and *IGF-1R*^{present} MMC (Figure 7A-D). Due to the low number of patients with spiked *MMSET* and *IGF-1R*^{absent} MMC (respectively 6 and 3 in the 2 series) and of patients with spiked *MMSET* and *IL-6R*^{low} (respectively 6 and 9 in the two series), their survival could not be evaluated. High *IL-6R* expression without spiked *MMSET* had prognostic value for OAS compared to patients with *IL-6R*^{low} MMC in the LR-TT2 series only. Patients with spiked MMSET and *IL-6R*^{high} MMC had decreased EFS and OAS compared to patients with *IL-6R*^{high} MMC and without spiked *MMSET* in the 2 series (Figure 7E-H). Using univariate Cox analysis, *IGF-1R*^{absent} MMC or spiked *MMSET* and *IGF-1R*^{presence} MMC had prognostic value for EFS and OAS in the 2 patient series. MMC with *IGF-1R*^{presence} and lacking spiked *MMSET* had no prognostic value. Using multivariate Cox analysis, none of the 3 parameters had prognostic value for either EFS or OAS in the 2 series (results not shown).

Discussion

We selected 5 documented MGF for which recombinant MGF and inhibitors are commercially available to define a hierarchy of their biological action on HMCLs. We have found that IGF-1 is the major MGF in agreement with several studies[9,41], IL-6 an important one, and that HGF, EGF family and BAFF/APRIL act on a subset of HMCLs only. In serum-free cultures, only the 3 $CD45^-$ HMCLs could survive within 4-6 days of culture through an autocrine IGF-1/IGF-1R loop. These cells coexpressed *IGF-1R* and *IGF-1* genes and IGF-1R and IGF-1 proteins and the NVP-AEW541 IGF-1R inhibitor, unlike other MGF inhibitors, abrogated their survival. Regarding $CD45^+$ HMCLs, although an autocrine IGF-1/IGF-1R loop was present in 4/5 HMCLs, it was not sufficient to promote survival. But this autocrine IGF-1/IGF-1R loop was necessary for the growth activity of IL-6, HB-EGF or HGF when MMC expressed IGF-1R. Adding a high concentration of IL-6 (up to 30 ng/mL) could not rescue from apoptosis due to IGF-1 pathway inhibition (data not shown). The specificity of NVP-AEW541 for IGF-1R targeting was previously reported [35] and is emphasized here by its lack of effect on the $IGF\text{-}1R^-$ XG-12 HMCL and its lack of inhibition of IL-6 or HGF-induced transduction signals. IL-6 increases proliferation of 7/8 HMCLs tested, but interestingly its effect is dependent on the presence of an autocrine IGF-1/IGF-1R loop when MMC expressed IGF-1R. IGF-1 is detected by western blot in myeloma cells but could not be detected in HMCL culture supernatant. This does not preclude a bioactive role of autocrine IGF-1 since the bioactive concentration of rIGF-1 on HMCLs (27 pg/mL) is below the detection limit of commercially available IGF-1 ELISA (≥ 45 pg/mL). In addition, the survival of the $CD45^-$ HMCLs and the IL-6-induced stimulation of $CD45^+$ HMCLs in serum-free medium are also blocked by recombinant IGF-binding protein 3 (IGFBP-3), another IGF-1 inhibitor (results not shown). To

study the cooperation between IL-6 and IGF-1, different techniques have been used focussing on different aspects that may yield to challenging conclusions [10,25,26]. Our current data did not confirm a previous study showing that the IL-6-induced growth of $CD45^+$ HMCLs was not inhibited by an IGF-1R inhibitor [26]. An explanation may be the use of foetal calf serum containing medium, which comprises IGF-1 but also insulin that stimulates MMC growth [42]. We used here a serum-free culture medium, devoid of insulin, making it possible to unravel this major role of autocrine IGF-1. This matter is of great importance in view of anti-IGF-1 therapy. Indeed, the report by Descamps *et al.* suggest that an anti-IGF-1R MoAb therapy will be unable to target $CD45^+$ MMC, that include the proliferating MMC [26]. On the contrary, our data suggest that an IGF-1R inhibitor therapy could be useful in patients with $IGF\text{-}1R^{present}$ MMC, independently of CD45 expression. Only 2/8 HMCLs were stimulated by HGF although *c-Met* is expressed by 7/8 HMCLs. Another HMCL is stimulated by HB-EGF, whereas 8/8 HMCLs expressed at least one of the 4 ErbB receptors [43]. These effects were abrogated by the specific inhibitor of HGF or HB-EGF and also by the IGF-1R inhibitor, but not the anti-IL-6 MoAb, BCMA-FC and pan-ErbB kinase inhibitor (for HGF effect) or anti-HGF MoAb (for HB-EGF effect). Thus targeting IGF-1R could also help to block their activity. Only APRIL-activity is not affected by IGF-1R inhibition. Out of the 3 BAFF/APRIL receptors - *BAFF-R, TACI, BCMA* - MMC expressed always *BCMA*, *TACI* in one third of HMCLs, and rarely *BAFF-R* [44].

These in vitro data fit well with the prognostic value of receptor expression of these 5 MGF on MMC since only *IGF-1R and IL-6R* expression have prognostic value using 2 independent patient series. *IGF-1R* gene is not expressed by normal B and plasma cells, including plasmablastic cells. Thus *IGF-1R* is aberrantly expressed by 31%-50% of MMC of previously-untreated patients. Of note, 90% of HMCLs expressed

IGF-1R. HMCLs are mainly obtained from patients with extramedullary proliferation [31,45] and this increased frequency of *IGF-1R*^{presence} in HMCLs compared to that in primary MMC may reflect an increase frequency of *IGF-1R*^{present} MMC in patients with extramedullary proliferation. Alternatively, it might be due to the way of obtaining HMCLs using culture medium and serum that contain large amount of circulating IGF-1, thus favoring the growth of *IGF-1R*^{present} MMC.

Presently, no conclusive data have been published regarding the prognostic value of IGF-1R on MMC [29,30]. We have shown here that *IGF-1R* expression is prognostically significant in two independent large sets of patients obtained in two centers, using different methods for the Affymetrix probe preparation (single or double in vitro transcription amplification) and two different Affymetrix platforms [3,46]. The poor prognosis of patients with *IGF-1R*^{present} MMC is not only explained by a strong association of *IGF-1R*^{present} MMC and poor prognosis t(4;14) translocation and spiked MMSET expression [1]. Indeed, patients with *IGF-1R*^{present} MMC and unspiked *MMSET* had also a significantly shorter survival than patients with *IGF-1R*^{absent} MMC. This might be explained by the increased proportion of patients with *IGF-1R*^{present} MMC in the poor prognosis proliferation group (75.9% versus 49.9%) [3] and in patients with del17, another poor prognosis abnormality [1] that occurs independently of t(4;14). Noteworthy, we show here that IGF-1 is a major factor driving the proliferation of MMC, which could account for the proliferation signature. Patients with both *IGF-1R*^{present} MMC and t(4;14) had the shortest survival. A possible explanation is that patients with t(4;14) need to acquire additional aberrations (e.g. aberrant expression of *IGF-1R*) for the outbreak of overt MM.

MMC are “bathed” in high levels of IGF-1 in the tumor milieu in vivo. First, IGF-1 is directly produced in the bone marrow, by MMC and by osteoclasts. In addition, high

levels of IGF-1 - bound to IGFBP-3 and ALS protein - circulate in patients with MM and healthy individuals [47] and serum levels of IGF-1 correlated with poor prognosis in patients with MM [28]. These circulating IGF-1-IGFBP-3-ALS complexes can be captured by MMC that expressed highly syndecan-1, that bind IGFBP-3 [47]. IGFBP-3 binding to heparan sulfate chains weakens its affinity with IGF-1, which is thus able to bind membrane IGF-1R and exert its biological activity. In addition, MMC produce soluble syndecan-1, in particular though an heparanase controlled process [46,48,49], providing an extracellular matrix able to bind circulating IGF-1-IGFBP complexes and to release IGF-1 close to MMC.

IL-6R is variably expressed in MMC of all patients with MM. Dividing MM patients within two groups using *IL-6R* median expression, we found that patients with *IL-6R^{high}* MMC had a shorter survival. This might be explained by the increased proportion of patients of poor prognosis groups (proliferation, MAF and MMSET groups) [3] in *IL-6R^{high}* group. Patients with both *IL-6R^{high}* MMC and t(4;14) had a worst survival.

A message of this study is not that *IGF-1R* expression can be useful to define new prognostic classification, as the adverse prognosis value of *IGF-1R* expression is explained mainly by their expression in already identified poor prognosis groups, *i.e.* t(4;14), del17 and proliferation groups. But a message is that the adverse prognosis value of *IGF-1R* expression in MMC together with its major MGF activity emphasize that targeting IGF-1 could be promising for the treatment of patients with MM. A phase I study of anti-IGF-1R antibody therapy in patients with refractory MM was recently reported [50]. This trial showed no toxicity and disease stabilization in about half of the patients. Since *IGF-1R* is present on MMC of 30% to 50% of the newly-diagnosed patients, *IGF-1R* expression on MMC should be evaluated in patients

treated with anti-IGF-1 therapy. Anti-IL-6 MoAb treatment was also shown to block MMC proliferation with temporary disease stabilization [51]. Thus, anti-IL-6 therapy could be a useful combination with an IGF-1 inhibitor.

In conclusion, this study makes it possible to define a hierarchy of the biological action of 5 well-documented MGF on HMCLs, IGF-1 being the major one, IL-6 an important one, and HGF, EGF family and BAFF/APRIL acting only on a subset of HMCLs. Of interest, this hierarchy of biological activity of these 5 MGF using HMCLs fully paralleled with the prognostic value of the expression of the genes of the receptors of these MGF in MMC since *IGF-1R* and *IL-6R* expressions in MMC had prognostic value. Thus, gene expression profiles of MMC and of the tumor environment is highly recommended for a better understanding and anticipation of the efficacy of growth factor targeted therapy in patients with MM.

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Author contributions:

ACS designed research, performed the experiments and wrote the paper.
DH, AS, JM, MH, TM, MJ, TM, AJ, KM, UB, JFR and HG collected bone marrow samples and clinical data.
LC provided some new reagents.
TR and AK participated in the analyzing of the data.
JS and BB provided GEP and patient's data and participated in the writing of the paper.
DH and HG participated in the writing of the paper.
BK is the senior investigator who designed research and wrote the paper.

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Categories	*IGF-1R*		*IL-6R*	
	IGF-1R^{absent} **(n = 118)**	***IGF-1R^{present}*** **(n = 53)**	***IL-6R^{low}*** **(n = 85)**	***IL-6R^{high}*** **(n = 85)**
	% of patients in each group		% of patients in each group	
Age ≥ 65 yr	26%	21%	26%	24%
Kappa light chain	64%	64%	63%	65%
Lambda light chain	33%	34%	36%	32%
Non-secreting	3%	2%	1%	3%
B2M ≤ 3.5 mg/ml	60%	58%	63%	56%
B2M > 5.5 mg/ml	17%	17%	19%	15%
IgA subtype	***20%***	***33%***	***15%***	***33%***
LDH ≥ 240 IU/liter	***26%***	***12%***	24%	19%
Albumin < 35 g/l	37%	36%	44%	31%
Hemoglobin < 10 g/dl	31%	32%	29%	34%
C-reactive protein ≥ 5 mg/l	40%	40%	43%	38%
Bone lesions				
0: normal bone structure	18%	27%	21%	22%
1: osteopenie / osteoporosis	28%	25%	22%	33%
2: osteolyse [1-3]	8%	10%	9%	9%
3: major structural damage [>3]	46%	38%	48%	36%

	IGF-1R^{absent} **(n = 118)**			***IGF-1R^{present}*** **(n = 53)**		
Staging	I	II	III	I	II	III
ISS	44%	36%	20%	42%	42%	16%
	IL-6R^{low} **(n = 85)**			***IL-6R^{high}*** **(n =85)**		
Staging	I	II	III	I	II	III
ISS	44%	35%	21%	42%	41%	17%

Table 1: Clinical data of patients with *IGF-1R^{absent}* and *IGF-1R^{present}* MMC and of patients with *IL-6R^{low}* and *IL-6R^{high}* MMC.

The 171 previously-untreated patients with MM were treated at the university hospitals of Heidelberg of Montpellier. Patients were separated in two groups: patients with *IGF-1R^{absent}* MMC and patients with *IGF-1R^{present}* MMC or patients with *IL-6R^{low}* MMC and *IL-6R^{high}* MMC, as assayed with Affymetrix microarrays. Data are the percentages of patients within these 2 groups with the indicated clinical or biological parameters. When the percentages were different with a Chisquare test ($P \leq .05$), data are shown in bold and italic. NS: not significant.

	t(4;14)$^{+}$ (n = 20)	t(4;14)$^{-}$ (n = 74)		t(4;14)$^{+}$ (n = 20)	t(4;14)$^{-}$ (n = 74)
IGF-1R^{present}	***70%***	***26%***	***IL-6R^{high}***	***85%***	***53%***
IGF-1R^{absent}	***30%***	***74%***	***IL-6R^{low}***	***15%***	***47%***
	del13^{+} (n = 71)	del13^{-} (n = 59)		del13^{+} (n = 71)	del13^{-} (n = 59)
IGF-1R^{present}	***45%***	***19%***	***IL-6R^{high}***	***66%***	***40%***
IGF-1R^{absent}	***55%***	***81%***	***IL-6R^{low}***	***34%***	***60%***
	del17^{+} (n = 24)	del17^{-} (n = 97)		del17^{+} (n = 24)	del17^{-} (n = 97)
IGF-1R^{present}	***58%***	***27%***	***IL-6R^{high}***	46%	58%
IGF-1R^{absent}	***42%***	***73%***	***IL-6R^{low}***	54%	42%
	1q21^{+} (n = 48)	1q21^{-} (n = 66)		1q21^{+} (n = 48)	1q21^{-} (n = 66)
IGF-1R^{present}	***42%***	***27%***	***IL-6R^{high}***	***67%***	***47%***
IGF-1R^{absent}	***58%***	***73%***	***IL-6R^{low}***	***33%***	***53%***
	t(11;14)$^{+}$ (n = 15)	t(11;14)$^{-}$ (n = 104)		t(11;14)$^{+}$ (n = 15)	t(11;14)$^{-}$ (n = 104)
IGF-1R^{present}	33%	33%	***IL-6R^{high}***	***40%***	***57%***
IGF-1R^{absent}	67%	67%	***IL-6R^{low}***	***60%***	***43%***

Table 2: Genetic abnormalities of patients with *IGF-1R^{absent}* and *IGF-1R^{present}* MMC and of patients with *IL-6R^{low}* and *IL-6R^{high}* MMC.

Interphase-FISH-analysis was performed on CD138-purified plasma cells for 79 to 129 patients of the HM series. Patients were separated in two groups: patients with *IGF-1R^{absent}* MMC and patients with *IGF-1R^{present}* MMC or patients with *IL-6R^{low}* MMC and *IL-6R^{high}* MMC, as assayed with Affymetrix microarrays. Data are the percentages of patients within these 2 groups with the biological parameters. When the percentages were different with a Chisquare test ($P \leq .05$), data are shown in bold and italic. NS: not significant.

	all patients	PR	LB	MS	HY	CD1	CD2	MF	MY
Patient subgroups	100%	8.4%	9.0%	12.2%	18.8%	6.4%	11.9%	6%	27.5%
***Frequency of* patients with *IGF-1R*^{present}**	49.9%	***75.9%***	61.3%	***92.9%***	***33.8%***	***22.7%***	43.9%	55%	37.9%
***Frequency of* patients with *IL-6R*^{high}**	50%	***82.8%***	***93.5%***	***78.6%***	38.5%	***9.1%***	***17.1%***	***75.0%***	39.4%

Table 3: *IGF-1R* and *IL-6R* expressions in MMC according to the molecular classification of multiple myeloma.
The percentage of patients with *IGF-1R*^{present} and *IL-6R*^{high} MMC was determined in the 8 patient subgroups defined by Zhan *et al*[3]. PR: proliferation. LB: low bone disease. MS: MMSET. HY: hyperdiploid. CD-1 and CD-2: CCND1/CCND3. MF: MAF/MAFB and MY: myeloid group. When the percentages of patients *IGF-1R*^{present} or *IL-6R*^{high} MMC in a subgroup were significantly different from that in all patients with a Chisquare test ($P \leq .05$). data are shown in bold and italic.

Figure Legends

Figure 1: Survival of myeloma cell lines in serum-free culture medium.

(A) HMCLs were starved for 2 hours and cultured for 4 days without cytokine in the Syn H serum-free culture medium. The cell concentration at the start of the culture was 2 x 10^5 cells/mL for all HMCLs. Results are the mean luminescent signals of a luciferase assay in 3 independent experiments 2 hours and 4 days after culture start. * the mean value is significantly different from that obtained at 2 hours using a Student *t* test for pairs ($P \leq .05$). (B) CD45 protein expression was determined by flow cytometry using murine anti-CD45RO, anti-CD45RA and anti-CD45RB MoAbs in the 3 autonomously-surviving HMCLs and 5 non-autonomously-surviving ones. The fluorescence intensity was set up in order to get a MFI between 3 and 5 with isotype-matched control antibodies. Results are the percentage of positive cells and in brackets, the mean fluorescence intensity of positive cells. These data are from one experiment representative of 3. (C) XG-5, XG-7 and XG-20 HMCLs were starved for 2 hours and cultured for 4 days without inhibitor (control) or with the anti-IL-6 MoAb (10 µg/mL) or the IGF-1R inhibitor (1 µM) or the pan-ErbB kinase inhibitor (1 µM) or the anti-HGF MoAb (25 µg/mL) or BCMA-Fc (10 µg/mL) in the Syn H culture medium. The cell concentration at the start of the culture was 10^5 cells/mL. Results are the mean percentages (± SD) of the luminescent signal of each group compared to that of the control group in 3 independent experiments. * the mean value was significantly different from that obtained with the control group using a Student *t* test for pairs ($P \leq .05$).

Figure 2: Specificity of MGF inhibitors and IGF-1 production by HMCLs.

(A) XG-2 cells were starved at 37°C for 18 hours in Syn H serum-free culture medium. Cells were then cultured without cytokine (control) or with either IL-6 (200

pg/mL) or IGF-1 (100 ng/mL) or HGF (20 ng/mL) and without inhibitor or with anti-IL-6 MoAb (10 µg/mL) or IGF-1R inhibitor (1 µM) or anti-HGF MoAb (25 µg/mL) for 20 minutes at 37°C in the Syn H culture medium. The receptor kinase inhibitors were added to the cells for 4 hours at the end of starvation culture and during exposure to rMGF. The anti-MGF antibodies were preincubated with the rMGF for 1 hour before to be added to cells. Cell lysates were immunoblotted with anti-phospho-Akt antibody and then reprobed with anti-akt antibody, anti-phospho-MAPK antibody and then reprobed with anti-MAPK, anti-phospho-Stat3 antibody and then reprobed with anti-Stat3 antibody. Anti-β actin was used as a loading control. (B) HMCLs were cultured for 2 days without cytokine in the Syn H serum-free culture medium. Cell lysates were immunoblotted with an anti-IGF-1 antibody. Anti-β actin was used as a loading control and the U266 HMCL as a negative control for IGF-1 production (no expression of *IGF-1* gene using Affymetrix microarrays).

(C) XG-2 cells were starved for 2 hours in Syn H serum-free culture medium and then cultured without cytokine (control) or with increased concentrations of rIGF-1 for 4 days. Results are the mean luminescent signals ± SD determined in sixplicate culture wells and are those of one experiment representative of 3. Data are expressed as percentage of the signal obtained without cytokine. * Mean value was significantly different from that obtained in the control group using a Student *t* test ($P \leq .05$).

Figure 3: Growth activity of 5 factors in 8 HMCLs.

HMCLs were starved for 2 hours and cultured without growth factor (group) or with either IL-6 (200 pg/mL) or IGF-1 (100 ng/mL) or HB-EGF (1 µg/mL) or HGF (200 ng/mL) or APRIL (200 ng/mL) for 4 days in the Syn H culture medium. The cell concentration were 2 x 10^5 cells/mL for XG-1, XG-2, XG-6, XG-12, XG-14 HMCLs and 10^5 cells/mL for XG-5, XG-7, XG-20 HMCLs. Results are the mean luminescent

signal determined in 6 replicate culture wells and are those from 1 out of 3 representative experiments. * The mean value was significantly different from that obtained in the control group using a Student *t* test ($P \leq .05$).

Figure 4: IGF-1R inhibitor inhibited the effect of IL-6, HGF and HB-EGF, unlike that of APRIL.

(A) HMCLs were starved for 2 hours and cultured without cytokine or with either IL-6 (200 pg/mL) or IGF-1 (100 ng/mL) or APRIL (200 ng/ml) or HGF (20 ng/mL) or HB-EGF (1 µg/ml) and without inhibitor or with anti-IL-6 MoAb (10 µg/mL) or IGF-1R inhibitor (1 µM) or pan-ErbB kinase inhibitor (1 µM) or anti-HGF MoAb (25 µg/mL) or BCMA-Fc (10 µg/mL) for 4 days in the Syn H culture medium. The cell concentration were 2 x 10^5 cells/mL for XG-1 and XG-2 HMCLs and 10^5 cells/mL for XG-5, XG-7 and XG-20 HMCLs. Results are the mean luminescent signals ± SD determined in sixplicate culture wells and are those of one experiment representative of 3. Data are expressed as percentage of the signal obtained with the growth factor. * Mean value was significantly different from that obtained in the control group using a Student *t* test ($P \leq .05$). XG-5 HMCL was only stimulated by IGF-1 (2.8-fold), XG-20 HMCL by IL-6 (2.9-fold) or IGF-1 (3.5-fold), XG-7 HMCL by IL-6 (2.2-fold), IGF-1 (2.3-fold) or HGF (2.7-fold), XG-1 HMCL by IL-6 (11-fold), IGF-1 (5-fold) or APRIL (5-fold) and XG-2 by IL-6 (11-fold), IGF-1 (17-fold), HGF (17-fold) or HB-EGF (2-fold).

(B) HMCLs were starved for 2 hours and cultured without cytokine or with IL-6 (200 pg/mL), or IGF-1 (100 ng/mL) and without inhibitor or with an anti-IL-6 MoAb (10 µg/mL) or an IGF-1R inhibitor (1 µM) for 4 days in the Syn H culture medium. Data are expressed as the mean percentage of the inhibition of the cytokine stimulation by the inhibitor in 3 independent experiments. When the percentages were different with

a Student *t* test for pairs ($P \leq .05$), data are shown in bold and italic. NS: not stimulated.

Figure 5: Gene expression profile of MGF receptors.

Expression of *IGF-1R, IL-6R, gp130, c-met*, *TACI,* and *BCMA* genes were determined with Affymetrix human U133 Plus 2.0 in six memory B cell (MBC), seven normal plasmablast samples (PPC), seven normal bone marrow plasma cell samples (BMPC), MMC of 123 patients with previously-untreated MM and 20 HMCLs. A white histogram indicates that the gene had an absent Affymetrix call in the sample.

Figure 6: Event-free survival and overall survival of patients with previously-untreated MM with *IGF-1R*^{absent} or *IGF-1R*^{present} MMC and with *IL-6R*^{low} and *IL-6R*^{high} MMC.

IGF-1R and *IL-6R* expressions were assayed in purified MMC with Affymetrix U133 microarrays. Patients from Heidelberg or Montpellier hospitals (HM series) were treated with high dose chemotherapy and autologous stem cell transplantation. 118 patients had *IGF-1R*^{absent} MMC and 53 *IGF-1R*^{present} MMC and 85 patients had *IL-6R*^{low} MMC and 85 *IL-6R*^{high} MMC. We used also patients' data from the Arkansas-Little Rock group (GEO accession number GSE2658). These patients from the Arkansas group were treated with total therapy 2 and termed for convenience LR-TT2 series. 114 patients had *IGF-1R*^{absent} MMC and 136 *IGF-1R*^{present} MMC and 172 patients had *IL-6R*^{low} MMC and 172 *IL-6R*^{high} MMC.

Event-free survival of *IGF-1R*^{absent} MMC and *IGF-1R*^{present} MMC patients from HM series (A) and from LR-TT2 series (B). Overall survival of *IGF-1R*^{absent} MMC and *IGF-1R*^{present} MMC patients from HM series (C) and from LR-TT2 series (D). The *P*-value was determined with a log-rank test.

Event-free survival of *IL-6R*^{low} MMC and *IL-6R*^{high} MMC patients from HM series (E) and from LR-TT2 series (F). Overall survival *IL-6R*^{low} MMC and *IL-6R*^{high} MMC patients from HM series (G) and from LR-TT2 series (H). The *P*-value was determined with a log-rank test.

Figure 7: Event-free survival and overall survival of patients with previously-untreated MM with *IGF-1R*^{absent} or *IGF-1R*^{present} with or without spiked *MMSET* MMC and with *IL-6R*^{low} and *IL-6R*^{high} with or without spiked *MMSET* MMC.

Spiked *MMSET* was determined with Affymetrix U133 microarrays in the patients from HM series and LR-TT2 series (see Materials and Methods). In HM series, 112 patients had *IGF-1R*^{absent} MMC, 33 *IGF-1R*^{present} without spiked *MMSET* MMC and 20 *IGF-1R*^{present} with spiked *MMSET* MMC. 79 patients had *IL-6R*^{low} MMC, 65 *IL-6R*^{high} without spiked *MMSET* MMC and 20 *IL-6R*^{high} with spiked *MMSET* MMC. In LR-TT2 series, 170 patients had *IGF-1R*^{absent} MMC, 126 *IGF-1R*^{present} without spiked *MMSET* MMC and 46 *IGF-1R*^{present} with spiked *MMSET* MMC. 163 patients had *IL-6R*^{low} MMC, 132 *IL-6R*^{high} without spiked *MMSET* MMC and 40 *IL-6R*^{high} with spiked *MMSET* MMC.

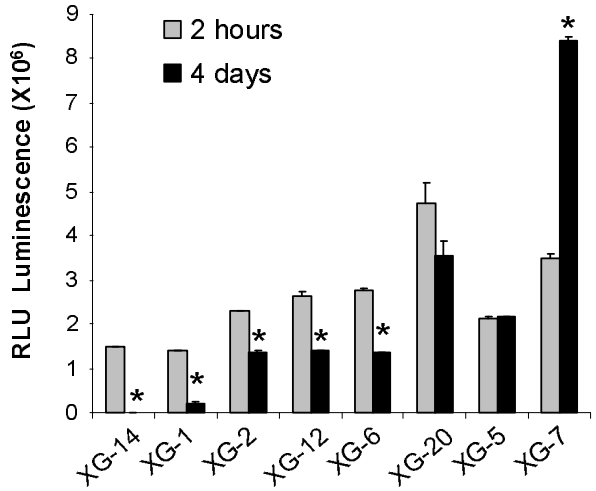
Event-free survival of *IGF-1R*^{absent} MMC and *IGF-1R*^{present} with or without spiked *MMSET* MMC patients from HM series (A) and from LR-TT2 series (B). Overall survival of *IGF-1R*^{absent} MMC and *IGF-1R*^{present} with or without spiked *MMSET* MMC patients from HM series (C) and from LR-TT2 series (D). The *P*-value was determined with a log-rank test.

Event-free survival of *IL-6R*^{low} MMC and *IL-6R*^{high} with or without spiked *MMSET* MMC patients from HM series (E) and from LR-TT2 series (F). Overall survival *IL-6R*^{low} MMC and *IL-6R*^{high} MMC with or without spiked *MMSET* patients from HM

series (G) and from LR-TT2 series (H). The *P*-value was determined with a log-rank test.

Figure 1

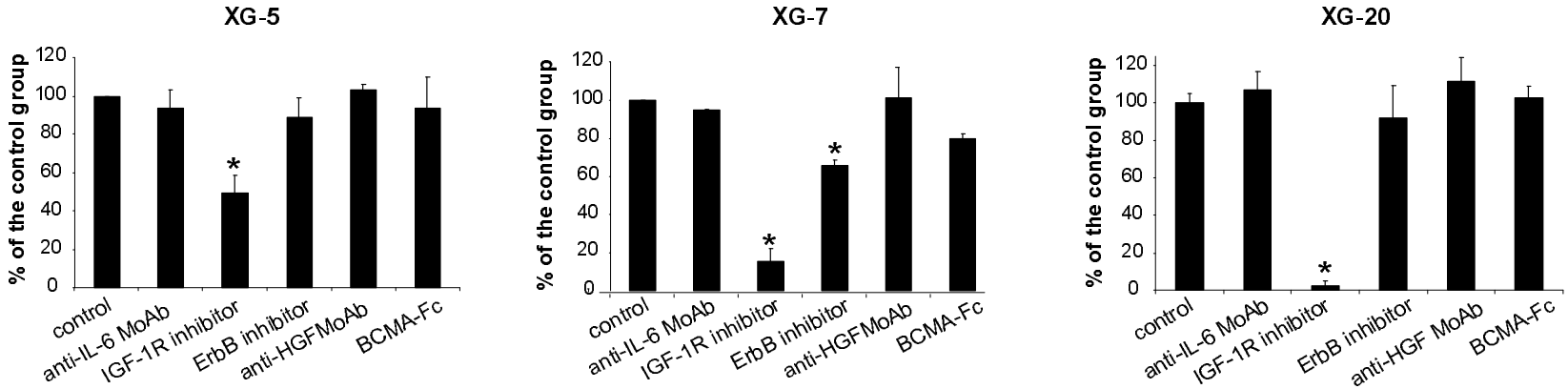
A

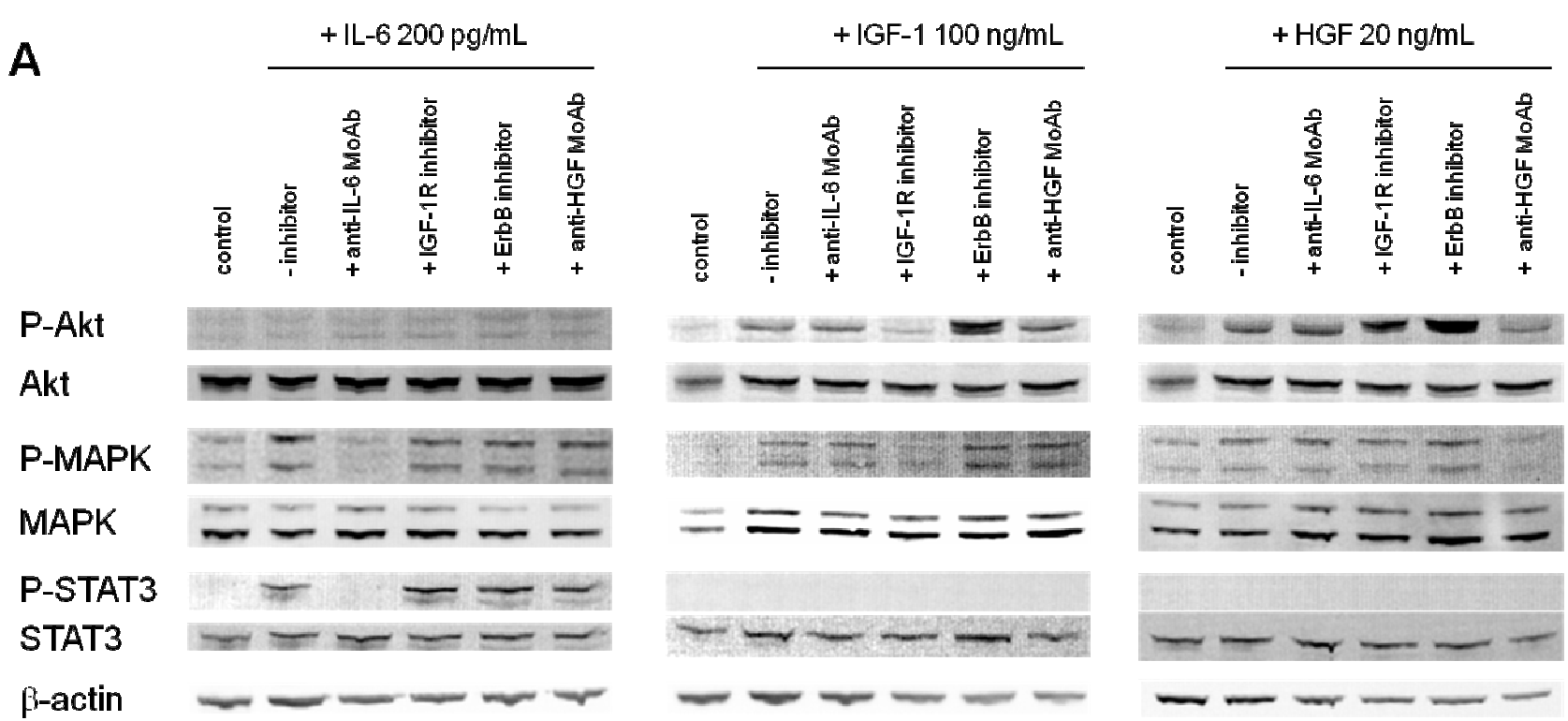


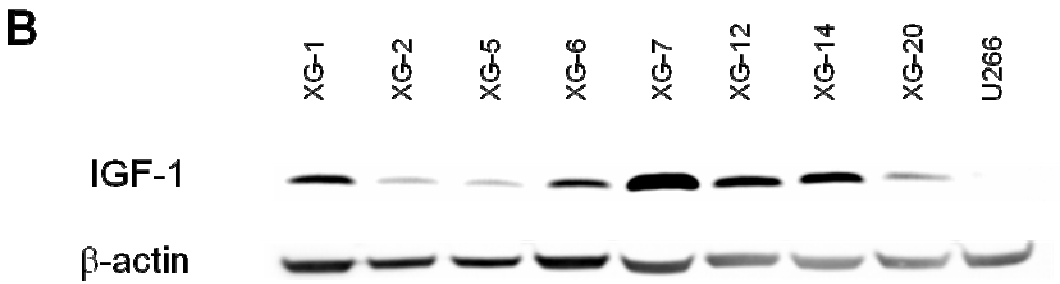
B

	Cell lines	CD45RO % (MFI)	CD45RA % (MFI)	CD45RB % (MFI)
Autonomously surviving HMCLs	XG-5	0.8 (37)	1.5 (17)	1 (17)
	XG-20	2 (20)	0.5 (13)	1 (4)
	XG-7	6 (23)	0.1 (15)	2 (10)
	Median Range	2 (23) 0.8-6 (23-37)	0.5 (15) 0.1-1.5 (13-17)	1 (10) 1-2 (4-17)
Non-autonomously surviving HMCLs	XG-2	34 (20)	16 (12)	3 (26)
	XG-14	75 (19)	25 (8)	91 (21)
	XG-1	97 (84)	87 (37)	87 (29)
	XG-6	100 (203)	24 (12)	97 (29)
	XG-12	100 (283)	98 (107)	100 (124)
	Median Range	97 (84) 34-100 (19-283)	25 (12) 16-98 (8-107)	97 (29) 3-100 (21-124)

C




A
+ IL-6 200 pg/mL
+ IGF-1 100 ng/mL
+ HGF 20 ng/mL
control
- inhibitor
+ anti-IL-6 MoAb
+ IGF-1R inhibitor
+ ErbB inhibitor
+ anti-HGF MoAb
P-Akt
Akt
P-MAPK
MAPK
P-STAT3
STAT3
β-actin


B
XG-1
XG-2
XG-5
XG-6
XG-7
XG-12
XG-14
XG-20
U266
IGF-1
β-actin

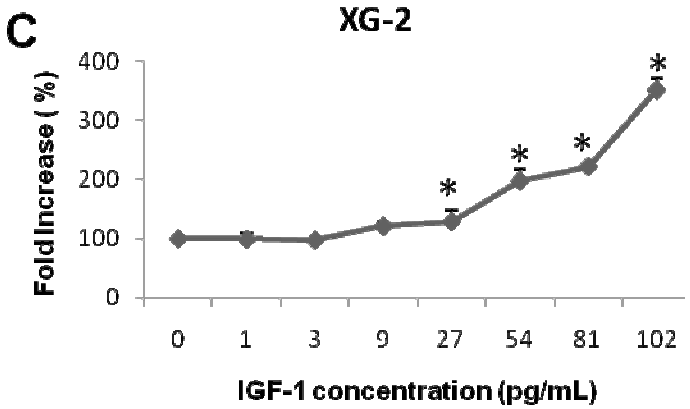
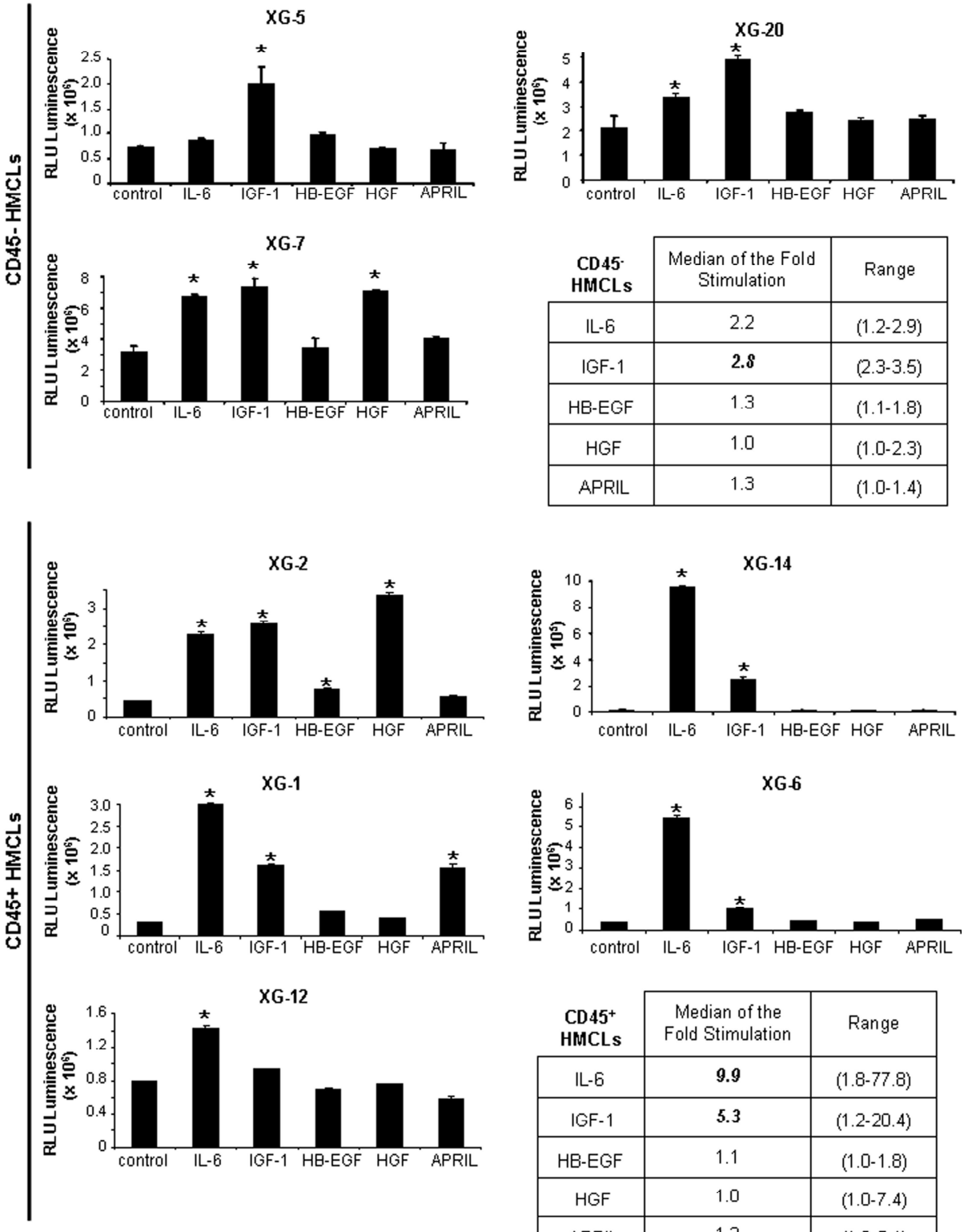

C
XG-2
Fold Increase (%)
400
300
200
100
0
0
1
3
9
27
54
81
102
IGF-1 concentration (pg/mL)

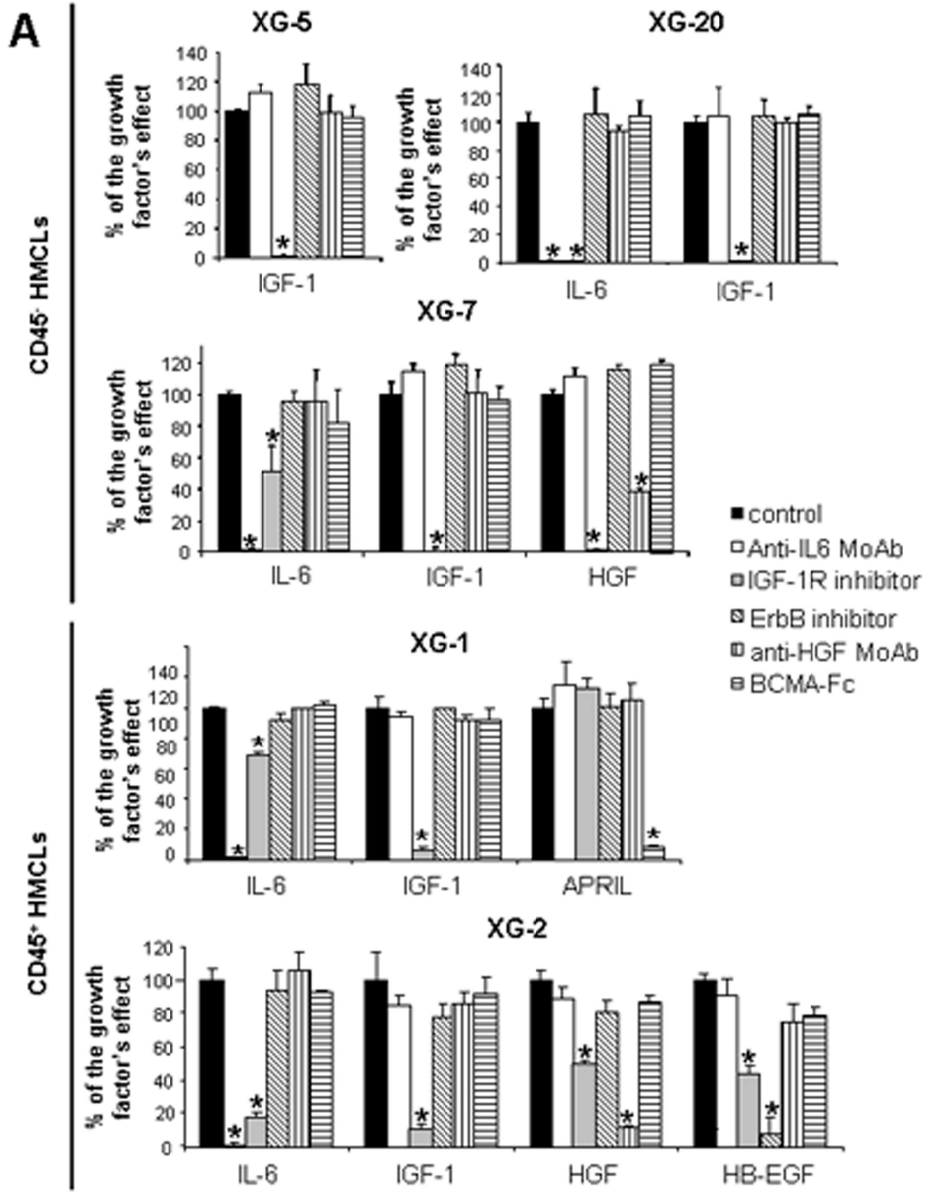
Figure 2

Figure 3



CD45⁻ HMCLs	Median of the Fold Stimulation	Range
IL-6	2.2	(1.2-2.9)
IGF-1	**2.8**	(2.3-3.5)
HB-EGF	1.3	(1.1-1.8)
HGF	1.0	(1.0-2.3)
APRIL	1.3	(1.0-1.4)

CD45⁺ HMCLs	Median of the Fold Stimulation	Range
IL-6	**9.9**	(1.8-77.8)
IGF-1	**5.3**	(1.2-20.4)
HB-EGF	1.1	(1.0-1.8)
HGF	1.0	(1.0-7.4)
APRIL	1.2	(1.0-5.1)



B

	Added growth factor	IL6	IGF-1	IL-6	IGF-1
		anti-IL6 MoAb % inhibition		IGF-1R inhibitor % inhibition	
CD45- HMCLs	XG-5	NS	0%	NS	*100%*
	XG-20	*100%*	0%	*99%*	*100%*
	XG-7	*100%*	0%	*49%*	*99%*
CD45+ HMCLs	XG-2	*91%*	15%	*81%*	*88%*
	XG-14	*100%*	0%	*98%*	*100%*
	XG-1	*100%*	6%	*31%*	*94%*
	XG-6	*99%*	12%	*65%*	*100%*
	XG-12	*100%*	NS	6%	NS
	Median	100%	0%	49%	100%
	Range	(91-100)	(0-15)	(6-99)	(88-100)

Figure 4

Figure 5

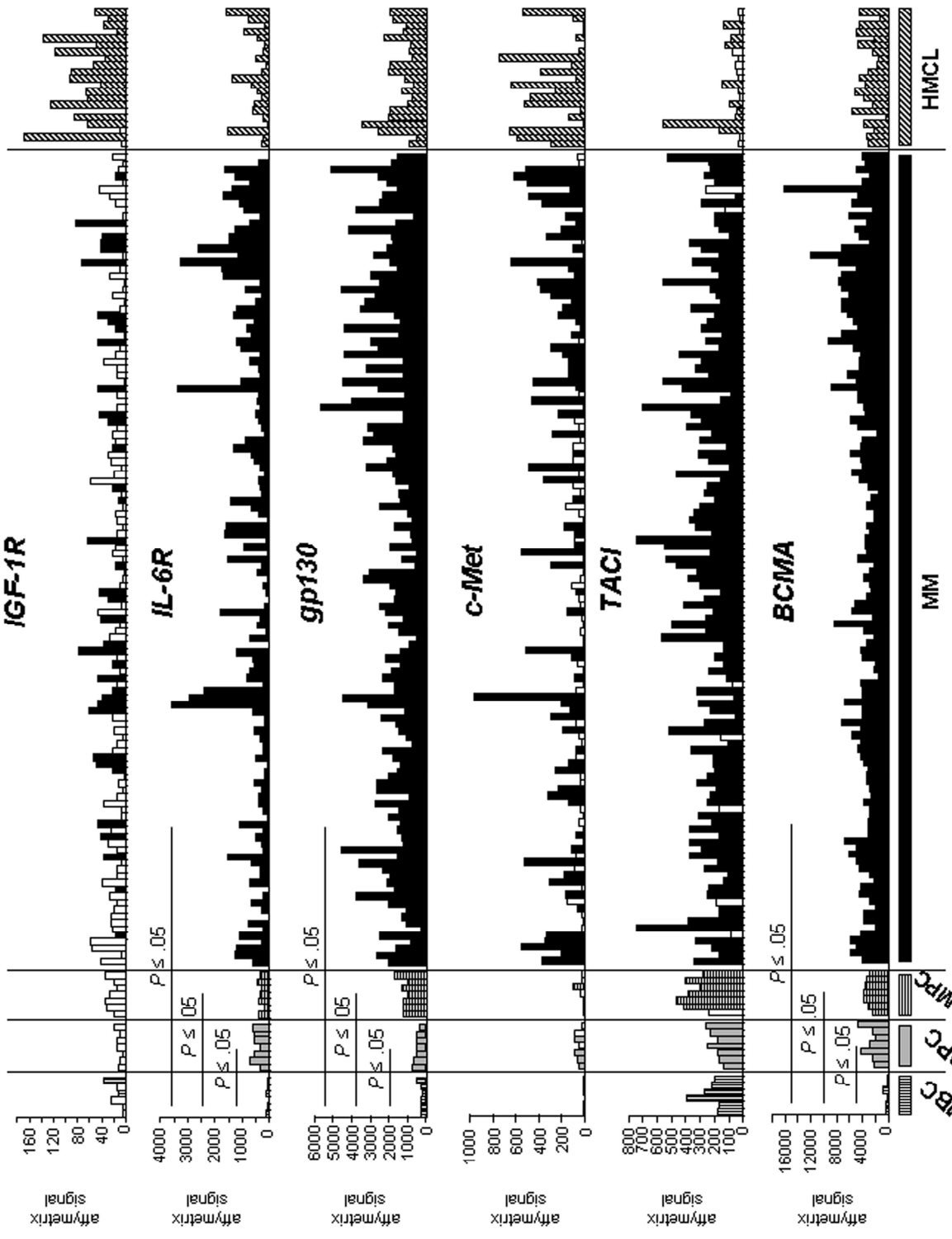

IGF-1R
IL-6R
gp130
c-Met
TACI
BCMA
aff/metrix signal
P ≤ .05
MBC
PPC
BMPC
MM
HMCL

Figure 6

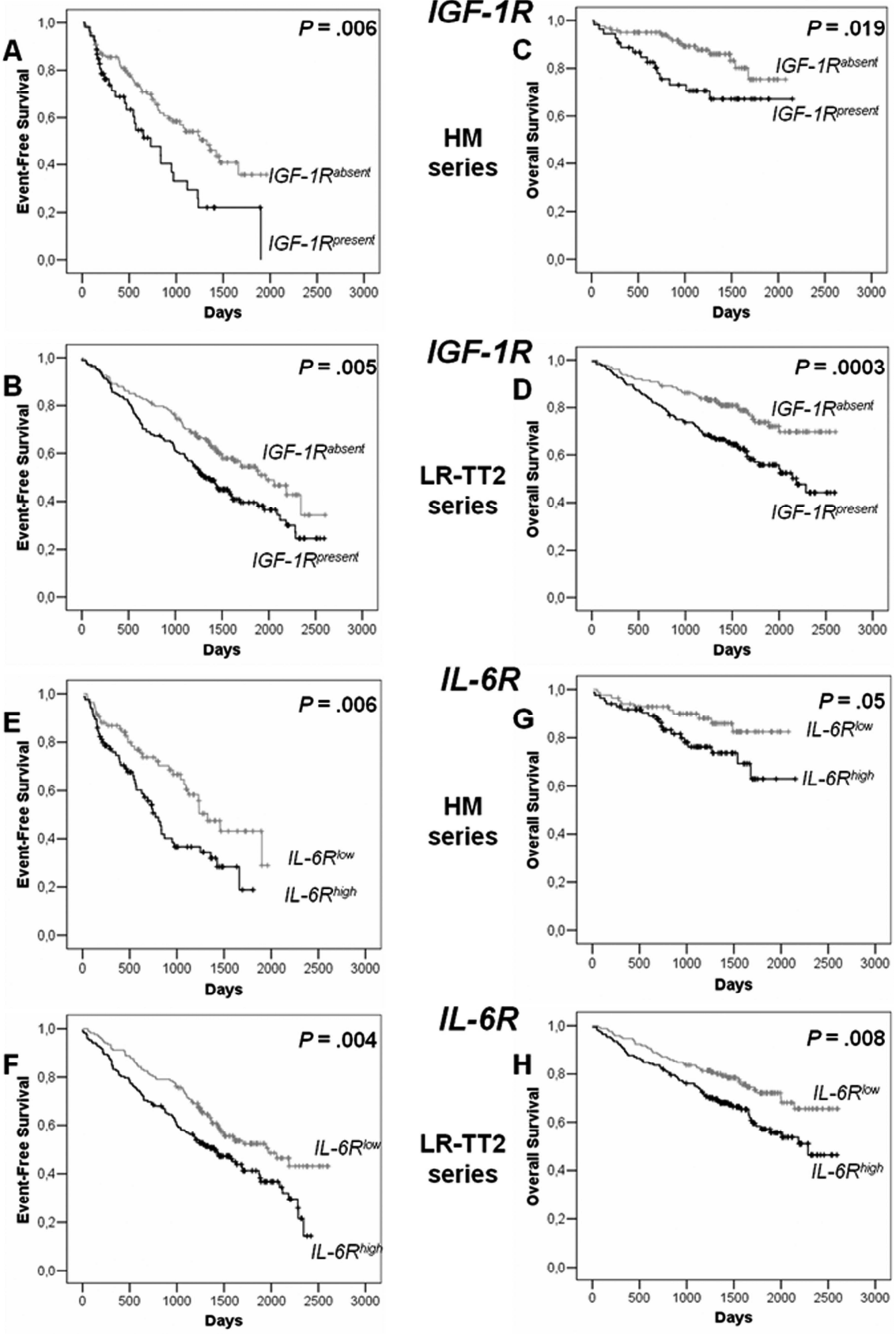
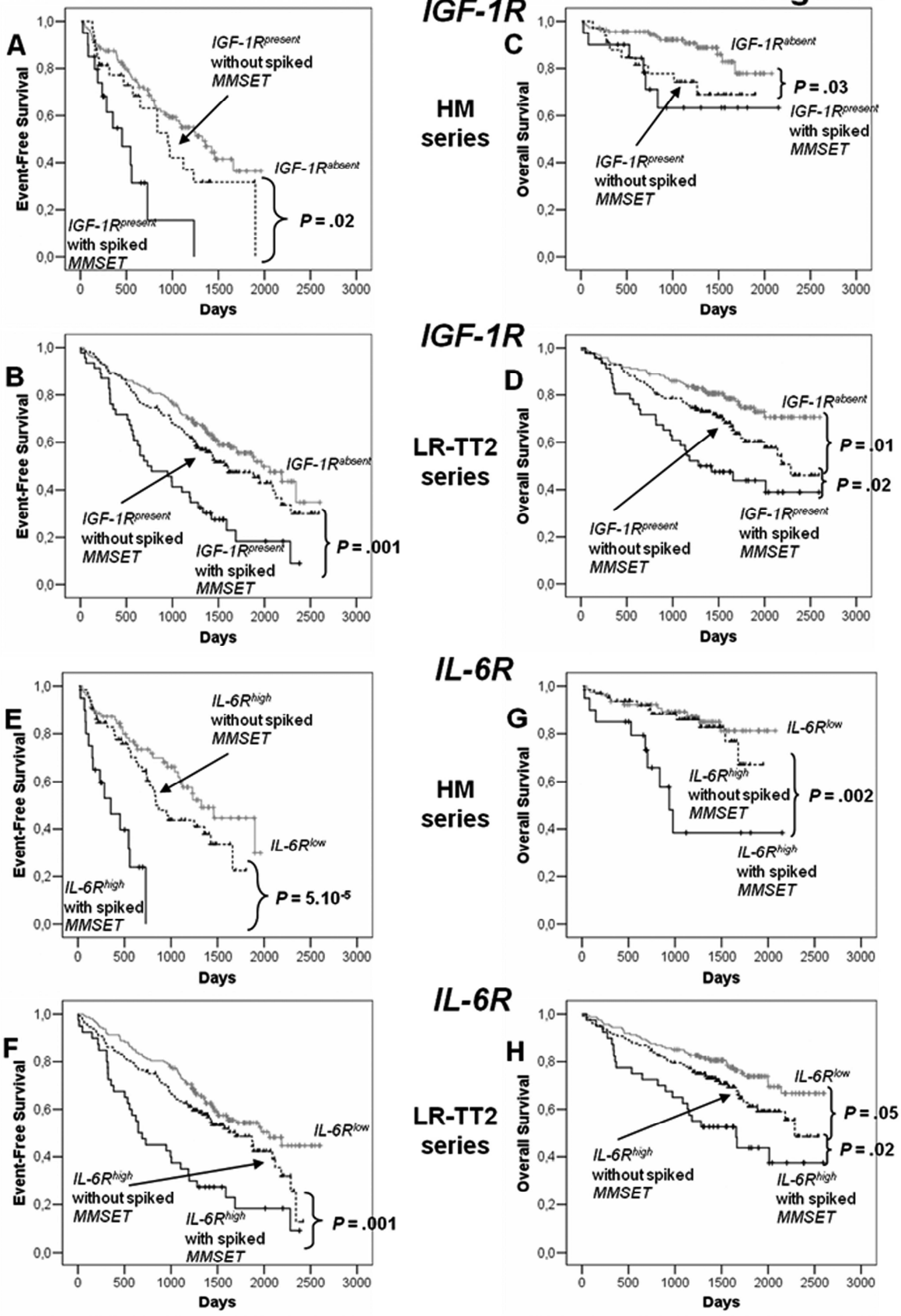

A
P = .006
Event-Free Survival
IGF-1R absent
IGF-1R present
Days
B
P = .005
Event-Free Survival
IGF-1R absent
IGF-1R present
Days
E
P = .006
Event-Free Survival
IL-6R low
IL-6R high
Days
F
P = .004
Event-Free Survival
IL-6R low
IL-6R high
Days
IGF-1R
C
HM series
P = .019
Overall Survival
IGF-1R absent
IGF-1R present
Days
IGF-1R
D
LR-TT2 series
P = .0003
Overall Survival
IGF-1R absent
IGF-1R present
Days
IL-6R
G
HM series
P = .05
Overall Survival
IL-6R low
IL-6R high
Days
IL-6R
H
LR-TT2 series
P = .008
Overall Survival
IL-6R low
IL-6R high
Days

Figure 7


A
Event-Free Survival
IGF-1R present without spiked MMSET
IGF-1R absent
IGF-1R present with spiked MMSET
P = .02
Days
B
Event-Free Survival
IGF-1R absent
IGF-1R present without spiked MMSET
IGF-1R present with spiked MMSET
P = .001
Days
IGF-1R
C
HM series
Overall Survival
IGF-1R absent
P = .03
IGF-1R present with spiked MMSET
IGF-1R present without spiked MMSET
Days
IGF-1R
D
LR-TT2 series
Overall Survival
IGF-1R absent
P = .01
P = .02
IGF-1R present without spiked MMSET
IGF-1R present with spiked MMSET
Days
E
Event-Free Survival
IL-6R high without spiked MMSET
IL-6R low
IL-6R high with spiked MMSET
P = 5.10-5
Days
F
Event-Free Survival
IL-6R low
IL-6R high without spiked MMSET
IL-6R high with spiked MMSET
P = .001
Days
IL-6R
G
HM series
Overall Survival
IL-6R low
IL-6R high without spiked MMSET
P = .002
IL-6R high with spiked MMSET
Days
IL-6R
H
LR-TT2 series
Overall Survival
IL-6R low
P = .05
P = .02
IL-6R high without spiked MMSET
IL-6R high with spiked MMSET
Days