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Jérôme Moreaux, Dirk Hose, Amélie Bonnefond, Thierry Rème, Nicolas Robert, Hartmut Goldschmidt, Bernard Klein

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Moreaux J*‡, Hose D°&, Bonnefond A‡, Reme T*‡, Robert N*, Goldschmidt H°&, Klein B*†‡

* CHU Montpellier, Institute of Research in Biotherapy, Montpellier, FRANCE;
‡ INSERM, U847, Montpellier, F-34197 France;
° Medizinische Klinik und Poliklinik V, Universitätsklinikum Heidelberg, Heidelberg, Germany
† Université MONTPELLIER1, UFR Médecine, Montpellier, France;
& Nationales Centrum für Tumorerkrankungen, Heidelberg, Germany

Corresponding author: Pr. Bernard KLEIN, PhD
Institute for Research in Biotherapy
CHU Montpellier, Hopital St Eloi
Av Augustin Fliche
34285 MONTPELLIER Cedex 5
FRANCE
Phone: +33 4 67 33 01 90
Fax: + 33 4 67 33 79 05
e-mail: bernard.klein@inserm.fr
http://irb.chu-montpellier.fr/

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Abstract

Objective

Multiple myeloma (MM) is a plasma cell neoplasm characterized by the accumulation of malignant plasma cells within the bone marrow. This disease still remains incurable, despite major treatment improvements. However, gene expression profiling of multiple myeloma cells (MMC) may lead to the identification of new therapeutic targets.

Methods

Using Affymetrix microarrays, we identified the overexpression of the *MYEOV* gene in MMC of 171 patients with newly-diagnosed multiple myeloma, compared to normal plasma cells.

Results

The *MYEOV* gene was present (Affymetrix call) in 79% of MMC and in 15% of normal plasma cells. *MYEOV* gene is not expressed in cells of the patients' bone marrow (BM) environment. The down-regulation of *MYEOV* gene reduced the growth of a $MYEOV^{present}$ myeloma cell line, unlike a $MYEOV^{absent}$ one. Patients with $MYEOV^{absent}$ MMC have an increased event free survival compared to patients with $MYEOV^{present}$ MMC, after high-dose therapy and stem cell transplantation and a trend for increased overall survival. In a Cox-proportional-hazard model, *MYEOV* expression in MMC is predictive for EFS for patients independently of ISS stage, t(4;14) translocation, albumin or B2M serum levels. A knock out of *MYEOV* significantly reduced the growth of MMC.

Conclusion

Thus, *MYEOV* expression is a prognositic factor for patients with MM, in part through a role of MYEOV in the control of MMC proliferation.

Keywords

Multiple Myeloma, prognostic factor, therapeutic target, tumor growth, gene expression profiling.

Introduction

Multiple myeloma (MM) is a plasma cell neoplasm characterized by the accumulation of malignant plasma cells within the bone marrow (BM). Several autocrine or paracrine factors can promote multiple myeloma cell (MMC) survival and proliferation [1-4] and inhibition of MMC growth factors may have clinical applications in combination with other drugs[5-7].

In order to identify new therapeutic targets in MM, we compared gene expression profiles (GEP) of MMC with those of normal plasma cells, normal plasmablasts and normal peripheral blood B cells. We identified that *MYEOV* gene (for Myeloma overexpressed gene) was expressed in malignant plasma cells in 79% of newly-diagnosed patients with MM. The *MYEOV* gene was originally isolated by the application of the NIH/3T3 tumorigenicity assay with DNA from a gastric carcinoma. The chromosomal region 11q13 is frequently associated with genetic rearrangements in a large number of human malignancies, including B cell malignancies[8-10] and overexpression of MYEOV is frequently observed in breast tumors and oral and esophagal squamous cell carcinomas[11]. Although MYEOV is expressed in a subset of human myeloma cell lines (HMCLs) with t(11;14)(q13;q32) translocation[12], MYEOV expression is rarely related with t(11;14) in MM[13]. Recently, Janssen et al. demonstrated *MYEOV* gene expression is transcriptionally silenced by a DNA-methylation mechanism in esophageal squamous cell carcinomas[14].

The presence of functional domains such as RNP-1 (motif typical of RNA binding protein) and the studies of the short hydrophobic regions and of the C-terminal leucine/isoleucine tail showed that MYEOV might be directed to the membrane[12]. Nevertheless, the biological role of MYEOV remains unclear. Recent studies showed

that MYEOV siRNA decreased proliferation of gastric cancer cells and colon cancer cell lines *in vitro*[15, 16].

In this study, we demonstrate that MMC of 79% of the patients with newly-diagnosed MM express *MYEOV* gene. For patients treated with high dose chemotherapy (HDC) and autologous hematopoietic stem cell transplantation (ASCT), *MYEOV* gene expression is a prognostic factor for EFS independent of ISS stage, HRS, t(4;14) translocation, albumin or β2M serum levels.

Materials and methods

Cell samples

XG human myeloma cell lines were obtained as described[17-20]. SKMM, OPM2, LP1 and RPMI8226 HMCLs were purchased from ATTC (LGC Promochem, France). MMC were purified from 171 patients with newly-diagnosed MM after written informed consent was given in accordance with the Declaration of Helsinki and IRB approval of 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. Patients' characteristics are indicated in Supplementary Table S1. The obtaining and purification of MMC, normal bone marrow (BM) plasma cells (BMPC), memory B cells, polyclonal plasmablasts, osteoclasts, BM stromal cell lines, BM CD34 cells, BM CD3 T cells, BM monocytes and BM polymorphonuclear neutrophils were performed as previously described[21]. We also used Affymetrix data of a cohort of 208 purified MMCs from previously untreated patients from the University of Arkansas for Medical Sciences (UAMS, Little Rock, AR). These patients were treated with total therapy 3[22] and termed in the following LR-TT3 series. These data are

publicly available through online Gene Expression Omnibus (Gene Expression Profile of Multiple Myeloma, accession number GSE2658. http://www.ncbi.nlm.nih.gov/geo/. Accessed June 1, 2006). Publicly available data from LR-TT2 cohort were not used because these patients were treated with thalidomide[23] and this information is not publicly available.

Preparation of complementary RNA (cRNA) and microarray hybridization

RNA was extracted using the RNeasy Kit (Qiagen, Hilden, Germany). Biotinylated cRNA was amplified with a double *in vitro* transcription reaction and hybridized to the Affymetrix HG U133 set of Gene Chips, according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Microarray data have been deposited in the ArrayExpress public database, under accession numbers E-MEXP-2360 for BMPC [24] and E-TABM-937 for B cells, PPC, MMC and BM environment samples.

Real-time RT-PCR

Total RNA was converted to cDNA using the Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). The assays-on-demand primers and probes and the *Taq*Man Universal Master Mix were used according to the manufacturer's instructions (Applied Biosystems, Courtaboeuf, France). The measurement of gene expression was performed using the ABI Prism 7000 Sequence Detection System and analyzed using the ABI PRISM 7000 SDS Software. For each primer, serial dilutions of a standard cDNA were amplified to create a standard curve, and values of unknown samples were estimated relative to this standard curve in order to assess the PCR efficiency. Ct values were obtained for GAPDH and the respective genes of interest during log phase of the cycle. Gene of interest levels were normalized to GAPDH for each sample (δCt = Ct gene of interest – Ct GAPDH) and compared with

the values obtained for a known positive control using the following formula $100/2^{\Delta\Delta Ct}$ where ddCt = dCt unknown – dCt positive control.

5-Aza-2' –deoxycytidine (5-azadC) treatment

The human MM cell lines XG-6, L363, and LP1 were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Cells (2 x 10^5/mL) were treated either with 0.5 μmol/L Aza-dC or with no drug (control) for 7 days.

Study of apoptosis

After 4 days of culture, cells were washed twice in PBS and apoptosis was assayed with FITC-conjugated annexin V labeling (Boehringer). Fluorescence was analyzed on a FACScan flow cytometer (Becton Dickinson).

Cell cycle analysis

DNA was stained with propidium iodide (PI). Cells were washed in PBS, suspended in 1 mL of 75% ethanol/25% water at room temperature for 2 minutes and washed again. 500 μl of PBS containing PI (40 μg/mL) and RNAse (100 μg/mL) (both from Sigma, St Louis, MO, USA) were added to each sample. Cells were incubated for 30 minutes at 37 °C and stored at 4 °C in the dark before analysis with a FACScan flow cytometer using Cell Quest software. The cell cycle was analyzed with the ModFit LT software (Verity Software House, Topsham, ME, USA).

Western blot analysis

Cells were lysed in 10 mM Tris-HCl (pH 7.05), 50 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate (NaPPi), 1% triton X-100, 5 μM $ZnCl_2$, 100 μM Na_3VO4, 1 mM DTT, 20 mM β-glycerophosphate, 20 mM p-nitrophenolphosphate (PNPP), 20 μg/ml aprotinin, 2.5 μg/ml leupeptin, 0.5 mM PMSF, 0.5 mM benzamidine, 5 μg/ml pepstatin, and 50 nM okadaic acid. Lysates were resolved on 12% sodium dodecyl sulfate-polyacrylamide by gel electrophoresis (SDS-PAGE) and transferred to a

nitrocellulose membrane (Schleicher and Schuell, Kassel, Germany). Membranes were blocked for 2 hours at room temperature in 140 mM NaCl, 3 mM KCl, 25 mM Tris-HCl (pH 7.4), 0.1% Tween 20 (TBS-T), 5% non-fat milk and human Ig (1 mg/ml), and then immunoblotted with a rabbit anti-MYEOV (Proteintech Group, Chivago, IL). 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. Blots were quantified by densitometry using acquisition into Adobe Photo Shop (Adobe Systems, San Jose, CA), and analyzing with the NIH Image software (National Institutes of Health, Bethesda, MD, USA).

siRNA transduction

The *MYEOV* siRNA smart pool (M-016553-00) (Dharmacon Inc, IL, USA) was transduced by electroporation (Amaxa, Köln, Germany) using nucleofaction. We also used Dharmacon's negative control siRNA (ON-TARGETplus siCONTROL Non-Targeting siRNA) as a control. RPMI8226, XG-7 and LP1 HMCLs were electroporated using respectively the T solution and programs T-001 or A-023 according to manufacturer's instructions[25]. After electroporation, HMCLs were cultured for 4 days in 96-well flat-bottom microtiter plates in serum-free culture medium Syn H (ABCell-Bio, Montpellier, France) without cytokine as previously described[3].

Statistical analysis

Gene expression data were normalized with the MAS5 algorithm and analyzed with our bioinformatics platforms (RAGE, http://rage.montp.inserm.fr/ and Amazonia,

http://amazonia.montp.inserm.fr/)[26, 27] or the SAM (Significance Analysis of Microarrays) software[28].

Survival curves were plotted using the Kaplan-Meier method. The statistical significance of differences in event free or overall survivals between groups of patients was estimated by the log-rank test. Univariate analyses were done to screen for prognostic variables by using Cox proportional hazards regression. The Cox model was also used for multivariate analysis to identify the most significant variables related to survival. A P value $\leq .05$ was considered significant in all statistical analyses. Statistical comparisons were done with R (http://www.r-project.org/) or SPSS11 (SPSS Chicago, IL) software. The biological functions and pathways encoded by a gene list was analyzed with Ingenuity software (Ingenuity® Systems, www.ingenuity.com).

Results and Discussion

MYEOV gene expression was investigated in purified MMC from 171 patients and 20 HMCLs using Affymetrix microarrays. Of the 5 *MYEOV* probe sets, the 227342_s_at probe set was the most strongly correlated with real time RT-PCR data (r = .86, *P* < .001) on the 20 HMCLs (Figure 1A). The use of present or absent Affymetrix call determined by the MAS5 algorithm was also validated for this probe set since only the 9 real-time RT-PCR^{+} HMCLs had an Affymetrix present call (Figure 1 A). In the following, the present and absent Affymetrix call of 27342_s_at probe set was used to combine microarray data from different patients' cohort performed with A+B or U133 plus 2.0 microarrays. *MYEOV* gene was expressed (present call) in 9/20 HMCLs and in 131 of 171 purified MMCs of newly-diagnosed patients.

MYEOV gene expression was also confirmed at protein level in 9 HMCLs and primary MMCs from 4 patients using western blot (Figure 1 B & C). Normalized MYEOV protein expression was significantly correlated with *MYEOV* Affymetrix expression (r = .9, *P* = .001) (Figure 1 B). The western blot analysis of MYEOV expression was not easy since only 2 antibodies are commercially available and they were not validated in previous peer-reviewed publications. The antibody we used provided a specific but weak signal after incubation with the anti-MYEOV antibody. *MYEOV* gene expression in primary MMCs was compared to that in normal BM plasma cells, normal plasmablasts or normal memory B cells. Whereas *MYEOV* 27342_s_at probe set had a present call (*MYEOV*^{present}) in 9/20 HMCLs and 131/171 primary MMCs, it had rarely a present call in BMPC (1/7) and was absent (*MYEOV*^{absent}) in normal plasmablasts (0/7) or memory B cells (0/7) (Figure 2). Of note, *MYEOV* probe set had a present call in purified plasma cells from 6/7 patients with MGUS. The expression of *MYEOV* gene, associated with poor prognosis, in

plasma cells from individuals with the premalignant phase of MM is appealing. Such observation was already done for CD200 that is not expressed in normal plasma cells, aberrantly expressed in MMCs in association with a poor prognosis and also expressed in MGUS plasma cells[29]. A likely explanation is that cancer disease is a multi hit disease. *MYEOV* overexpression could be one oncogenic hit that requires additional hits, occurring in myeloma cells, to be able to promote tumor formation and/or drug resistance.

MYEOV gene expression was investigated in the BM environment from patients with MM. *MYEOV* gene was not expressed in CD14 monocytes, CD15 polynuclear cells and CD3 T cells purified from the BM of 5 newly-diagnosed patients. It is also not expressed in 7 osteoclast samples, in BM stromal cells and in $CD34^+$ hematopoietic stem cells from 5 patients with MM (Figure 2 and Supplementary Table S3). These data were validated by real-time RT-PCR (Supplementary data, Figure S1).

MYEOV gene expression in MMCs delineates a subset of patients with specific clinical characteristics. The frequencies of patients with lambda-light chain MM, with C-reactive protein level ≥ 5 mg/liter, LDH ≥ 240 IU/liter, or chromosome 13 deletion are increased in patients with $MYEOV^{present}$ MMCs (Supplementary data Table S2). Patients with $MYEOV^{absent}$ MMC had a better event free survival (55 months) compared to patients with $MYEOV^{present}$ MMC (26 months) (Figure 3 A) and a trend for better overall survival (P = .06) (Figure 3 B). In a Cox-proportional-hazard model, the absence or presence of *MYEOV* (P = 0.01, hazard ratio = 1.9) and ISS-stage (P = 0.001, hazard ratio = 1.6) are independently predictive for EFS (P = 0.04 and P = 0.002 respectively) (Table 1). If *MYEOV* expression is tested together with classical prognostic factors, *i.e.* serum albumin and serum β2M, *MYEOV* expression (P = 0.04), β2M (P = 0.006) and albumin (P = 0.02) remain independent prognostic

factors. *MYEOV* expression (*P* = .05) is an independent prognostic factor of spiked *MMSET* expression, that is an indicator of t(4;14) translocation (*P* = .0001) [3] and of a spiked *c-MAF* expression, that is an indicator of t(14;16) translocation (*P* = .004) [30] (Table 1). We also looked for the prognostic value of *MYEOV* expression in MMCs in publicly available data from LR-TT3 series. *MYEOV* had a "present" call in MMCs of 73% of these patients. Patients with $MYEOV^{absent}$ MMCs had a significant better overall survival in the LR-TT3 cohort (*P* = .04) (Figure 3 C). EFS data for the LR-TT3 cohort were not publicly available. A comparison of *MYEOV* prognostic value with those of other prognostic factors (ISS, genetic abnormalities) could not be done because these data are not publicly available.

MYEOV expression was previously described in a subset of HMCLs with t(11;14)(q13;q32) translocation[12]. No correlation between *MYEOV* expression and t(11;14)(q13;q32) translocation was found in primary MMCs (Supplementary data Table S2). Specht*, et al* also reported that MYEOV expression is rarely related with t(11;14) in MM [13]. Since *MYEOV* has been shown to be transcriptionally silenced by a DNA-methylation mechanism in esophageal squamous cell carcinomas, [14], we investigated such an epigenetic regulation of *MYEOV* gene in MMCs. A treatment with 5-aza-2' –deoxycytidine of 2 $MYEOV^{absent}$ HMCLs (XG-6 and LP1) induced *MYEOV* expression without affecting that in the $MYEOV^{present}$ L363 HMCL (Figure 4).

Recently, a knock out of *MYEOV* RNA (siRNA) has been shown to decrease proliferation of gastric cancer cells and colon cancer cell lines *in vitro* [15, 16] suggesting a role of MYEOV in cancer proliferation and invasion. The shorter EFS and OAS in patients with $MYEOV^{present}$ MMC could be explained by a role of MYEOV in MMC proliferation. *MYEOV* siRNA downregulated *MYEOV* gene expression by 63% and 67% in RPMI 8226 and XG-7 HMCLs respectively and significantly reduced

the growth of these 2 HMCLs by 40% and 65% respectively (*P* = .006 and *P* = .001, n=5) (Figure 5A&C). The *MYEOV* siRNA could also knock down MYEOV protein (Figure 5B). The growth of the *MYEOV*^{absent} HMCL LP1 was not affected by the *MYEOV* siRNA (Figure 5C). *MYEOV* siRNA did not significantly induce apoptosis in RPMI 8226 and XG-7 HMCLs, but it blocked the cell cycle entry into the S phase (Figure 5D&E and Table 3).

In order to identify genes that are co-regulated with *MYEOV* gene, gene expression of *MYEOV*^{present} MMCs and *MYEOV*^{absent} MMCs of newly-diagnosed patients were compared using SAM supervised analysis. Probe sets with a present call in less then 3 out of all patients and a variation coefficient ≤ 100 were excluded from the analysis, yielding to 7073 probe sets. 25 unique genes were differentially expressed between *MYEOV*^{present} and *MYEOV*^{absent} MMCs (2 fold ratio, 1000 permutations and false discovery rate (FDR) < 5 %) (Table 2). *MYEOV*^{present} MMCs overexpressed *MAGE-A6* cancer testis antigen. In agreement with the bad prognostic factor of *MYEOV* expression and its control of MMC proliferation, *MAGE-A6* expression is associated with a shorter event free survival in patients with MM[31] and MAGE-A3/6 protein is associated with elevated proliferation in MMCs[32].

MYEOV^{present} MMCs expressed more weakly the CD81 tetraspanin. The *CD81* gene downregulation in *MYEOV*^{present} MMCs was validated by quantitative RT-PCR (Supplementary Figure S2). A low CD81 expression may be also involved in treatment resistance since CD81 can inhibit the adhesion, migration, invasion, and viability of MMCs [33]. The anti-myeloma effect of CD81/CD82 involves a down-regulation of Akt, activation of FoxO transcription factors and a decrease in active mTOR and mTOR/rictor[34]. *MYEOV*^{present} MMCs overexpressed *Homer1* gene. Homer1 may regulate the apoptotic response of MMCs to TRAIL activation, since

TRAIL activation kills Homer1-positive cells unlike Homer1-negative cells[35]. Homer1 is generally more expressed in cancer cells than their normal counterpart suggesting it may confer on tumor cells the possibility to be killed by TRAIL activation[35]. A comprehensive analysis of the biological functions or pathways of the proteins encoded by these 25 genes using Ingenuity did not reveal more information.

In conclusion, we have identified that *MYEOV* expression by MMC is a new factor of poor prognosis in patients with MM, in part through a role of MYEOV in the control of MMC proliferation.

		EFS	
	Prognostic variable	Proportional hazard ratio	P-value
Univariate COX analysis	MYEOV ISS	1.9 1.62	0.03 0.001
Multivariate Cox analysis	MYEOV ISS	1.58 1.66	0.04 0.002

		EFS	
	Prognostic variable	Proportional hazard ratio	P-value
Univariate COX analysis	MYEOV B2M Alb	1.9 1.10 1.82	0.03 0.001 0.006
Multivariate Cox analysis	MYEOV B2M Alb	1.88 1.1 1.67	0.04 0.006 0.02

		EFS	
	Prognostic variable	Proportional hazard ratio	P-value
Univariate COX analysis	MYEOV MS group	1.9 2.99	0.03 0.0001
Multivariate Cox analysis	MYEOV MS group	1.78 2.81	0.05 0.0001

		EFS	
	Prognostic variable	Proportional hazard ratio	P-value
Univariate COX analysis	MYEOV c-MAF	1.9 2.74	0.03 0.008
Multivariate Cox analysis	MYEOV c-MAF	2.00 2.96	0.02 0.004

Table 1. Multivariate proportional hazards analysis.

Genes overexpressed in *MYEOV*^{present} MMCs					
Chip	Probeset	Gene	Localization	Ratio	Description
U133P	214612_x_at	*MAGEA6*	Xq28	5.9	melanoma antigen family A; 6
U133P	223629_at	*PCDHB5*	5q31	4.9	protocadherin beta 5
U133P	227342_s_at	*MYEOV*	11q13	4.1	myeloma overexpressed gene (in a subset of t(11;14) positive multiple myelomas)
U133P	205413_at	*MPPED2*	11p13	3.5	metallophosphoesterase domain containing 2
U133P	235228_at	*KIAA1912*	2p16.1	2.9	KIAA1912 protein
U133P	1553698_a_at	*C1orf96*	1q42.13	2.9	chromosome 1 open reading frame 96
U133P	207191_s_at	*ISLR*	15q23-q24	2.9	immunoglobulin superfamily containing leucine-rich repeat
U133P	205968_at	*KCNS3*	2p24	2.6	potassium voltage-gated channel; delayed-rectifier; subfamily S; member 3
U133P	217147_s_at	*TRAT1*	3q13	2.5	T cell receptor associated transmembrane adaptor 1
U133P	242260_at	*MATR3*	5q31.2	2.4	Matrin 3
U133P	213793_s_at	*HOMER1*	5q14.2	2.3	homer homolog 1 (Drosophila)
U133P	225904_at	*C1orf96*	1q42.13	2.2	chromosome 1 open reading frame 96
U133P	203895_at	*PLCB4*	20p12	2.0	phospholipase C; beta 4
U133P	229363_at	---	---	2.0	CDNA FLJ32121 fis; clone PEBLM1000083

Genes overexpressed in *MYEOV*^{absent} MMCs					
Chip	Probeset	Gene	Localization	Ratio	Description
U133P	201131_s_at	*CDH1*	16q22.1	4.1	cadherin 1; type 1; E-cadherin (epithelial)
U133P	209602_s_at	*GATA3*	10p15	2.8	GATA binding protein 3
U133P	222116_s_at	*TBC1D16*	17q25.3	2.8	TBC1 domain family; member 16
U133P	1560652_at	---	---	2.7	MRNA; cDNA DKFZp686L0310 (from clone DKFZp686L0310)
U133P	227915_at	*ASB2*	14q31-q32	2.7	ankyrin repeat and SOCS box-containing 2
U133P	211648_at	*IGHG3*	14q32.33	2.6	Immunoglobulin heavy constant mu
U133P	212486_s_at	*FYN*	6q21	2.2	FYN oncogene related to SRC; FGR; YES
U133P	233255_s_at	*BIVM*	13q32-q33.1	2.1	basic; immunoglobulin-like variable motif containing
U133P	200675_at	*CD81*	11p15.5	2.1	CD81 antigen (target of antiproliferative antibody 1)
U133P	203761_at	*SLA*	8q22.3-qter|8q24	2.0	Src-like-adaptor
U133P	210105_s_at	*FYN*	6q21	2.0	FYN oncogene related to SRC; FGR; YES

Table 2 : 25 unique genes differentially expressed between *MYEOV*^{present} and *MYEOV*^{absent} MMCs of newly diagnosed patients (SAM analysis, 2 fold ratio, 1000 permutations and false discovery rate (FDR) < 5 %).

		G0-G1	S-Phase	G2-M	P value
MYEOV^{present} HMCL	XG-7 *CT* siRNA	40,67	35,97	23,36	0.0007
	XG-7 *MYEOV* siRNA	54,27	25,33	20,39	
	RPMI8226 *CT* siRNA	54,41	19,74	25,86	0.04
	RPMI8226 *MYEOV* siRNA	63,17	13,48	23,35	
MYEOV^{absent} HMCL	U266 *CT* siRNA	50,85	34,75	14,39	NS
	U266 *MYEOV* siRNA	50,95	34,45	14,6	

Table 3 : After electroporation with *MYEOV* siRNA or siRNA CT, RPMI8226, XG-7 and U266 myeloma cells were cultured at 10^5 cells/ml. Cells were recovered after 3 days of culture, DNA was labelled with PI and cells were analyzed on a FACScan apparatus. The percentage of cells in the G0-G1, S and G2-M phases of the cell cycle is indicated and was determined using the ModFit LT software. Results are representative of three experiments.

Figure legends

Figure 1. Validation of *MYEOV* expression.

(A) Gene expression of *MYEOV* in 20 HMCLs was assayed with real time RT-PCR and normalized with *GAPDH* expression. The arbitrary value of 100 was assigned to XG-7 positive control. The coefficient of correlation between Affymetrix and real-time RT-PCR values was determined.

(B) Expression level of MYEOV protein in HMCLs using western blot and correlation with MYEOV gene expression Affymetrix signal value. For each cell line, the ratios of MYEOV and beta actin proteins were determined in order to compare MYEOV protein expression between cell lines.

(C) MYEOV protein expression in purified primary myeloma cells using western blot analysis.

Figure 2. *MYEOV* gene expression in myeloma cells from patients with MM and normal cells.

Affymetrix *MYEOV* gene expression in normal memory B cells, normal polyclonal plasmablasts, normal BM plasma cells (BMPC), purified myeloma cells from patients with multiple myeloma (MM), human myeloma cell lines, bone marrow (BM) CD34 cells, BM stromal cells, purified BM CD15, CD14 and CD3 cells and osteoclasts.

Figure 3. Association of *MYEOV* gene expression and survival of newly-diagnosed patients with MM.

(A) Kaplan-Meier plot of the event-free survival in patients with $MYEOV^{present}$ and $MYEOV^{absent}$ MMCs in the HM cohort of 171 patients.

(B) Kaplan-Meier plot of the overall survival in patients with $MYEOV^{present}$ and $MYEOV^{absent}$ MMCs in our cohort of 171 patients.

(C) Kaplan-Meier plot of the overall survival in patients with *MYEOV*^{present} and *MYEOV*^{absent} MMCs in the LR-TT3 cohort of 208 patients from the University of Arkansas School of Medical Sciences.

Figure 4. Epigenetic regulation of MYEOV gene in MMCs.

Gene expression of *MYEOV* in XG-6, LP1 and L363 HMCLs was assayed with real time RT-PCR. Cells (2 x 10^5/mL) were treated either with 0.5 μmol/L Aza-dC or with no drug (control) for 7 days. For each experiment, the expression of MYEOV in myeloma cells was compared to that of untreated myeloma cells which was assigned an arbitrary value. Data are mean values of five independent experiments.

Figure 5. Inhibition of *MYEOV* gene expression and myeloma cell growth by a *MYEOV* siRNA.

(A) Inhibition of *MYEOV* gene expression by a *MYEOV* siRNA. For each experiment, the expression of *MYEOV* in myeloma cells treated with *MYEOV* siRNA was compared to that of myeloma cells treated with control siRNA, which was assigned the arbitrary value of 100. Results are the mean *MYEOV* gene expression in five independent experiments. * The mean value is statistically significantly different from that obtained with a non-targeting control siRNA (siRNA CT) using Student's *t*-test for pairs ($P \leq .05$).

(B) Inhibition of MYEOV protein expression in MMC using *MYEOV* siRNA. RPMI8226 myeloma cells were treated for 4 days with *MYEOV* or control siRNA and MYEOV protein was assayed by western blot.

(C) After electroporation with *MYEOV* siRNA or siRNA CT, LP1, RPMI8226 and XG-7 HMCLs were cultured in serum-free culture medium for 4 days. Results are the mean ± SD values of the RLU fluorescence determined on sextuplet culture wells. Results shown are representative of five independent experiments. * The mean value

is statistically significantly different from that obtained with siRNA CT using Student's *t*-test ($P \leq .05$).

(D) After electroporation with *MYEOV* siRNA or siRNA CT, RPMI8226 and XG-7 myeloma cells were cultured at 10^5 cells/ml. Cells were recovered after 3 days of culture and apoptotic cells were detected by annexin V staining. Results are those of one experiment representative of three.

(E) DNA was labelled with PI and cells were analyzed on a FACScan apparatus. The percentage of cells in the S phase of the cell cycle is indicated and was determined using the ModFit LT software. Results are from one experiment representative of three.

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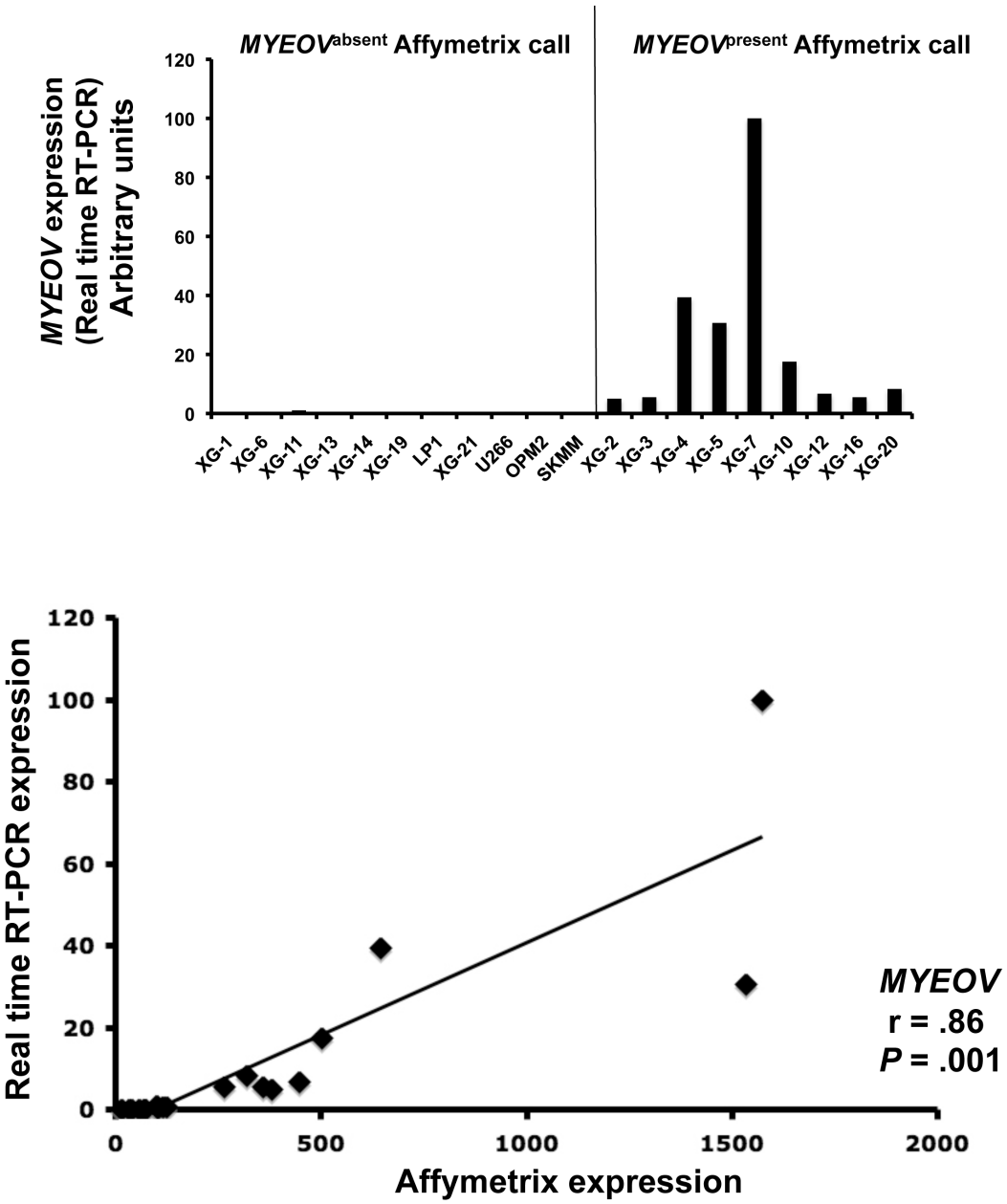
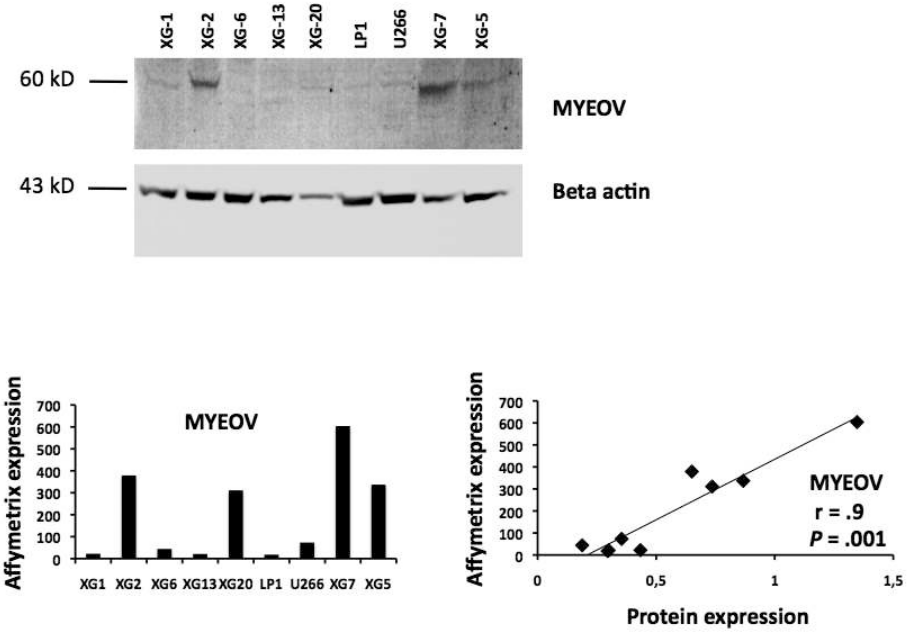

MYEOVabsent Affymetrix call
MYEOVpresent Affymetrix call
MYEOV expression
(Real time RT-PCR)
Arbitrary units
120
100
80
60
40
20
0
XG-1
XG-6
XG-11
XG-13
XG-14
XG-19
LP1
XG-21
U266
OPM2
SKMM
XG-2
XG-3
XG-4
XG-5
XG-7
XG-10
XG-12
XG-16
XG-20
Real time RT-PCR expression
120
100
80
60
40
20
0
0
500
1000
1500
2000
Affymetrix expression
MYEOV
r = .86
P = .001

Figure 1A

Figure 1 B


XG-1
XG-2
XG-6
XG-13
XG-20
LP1
U266
XG-7
XG-5
60 kD
MYEOV
43 kD
Beta actin
Affymetrix expression
MYEOV
700
600
500
400
300
200
100
0
XG1 XG2 XG6 XG13 XG20 LP1 U266 XG7 XG5
Affymetrix expression
MYEOV
r = .9
P = .001
0
0,5
1
1,5
Protein expression

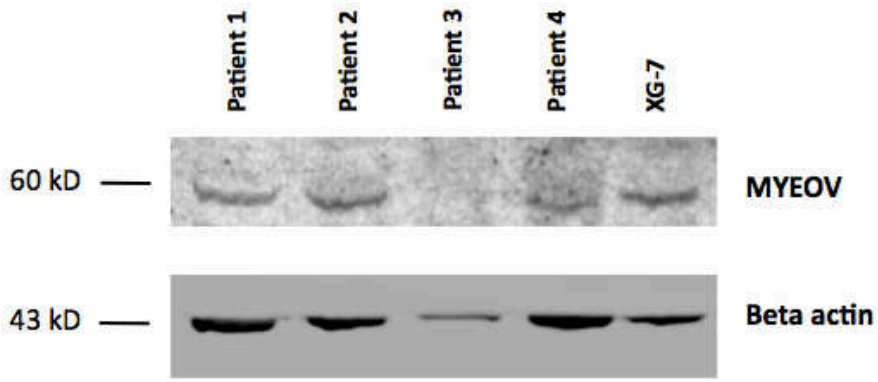

Patient 1
Patient 2
Patient 3
Patient 4
XG-7
60 kD
MYEOV
43 kD
Beta actin

Figure 1C

Figure 2

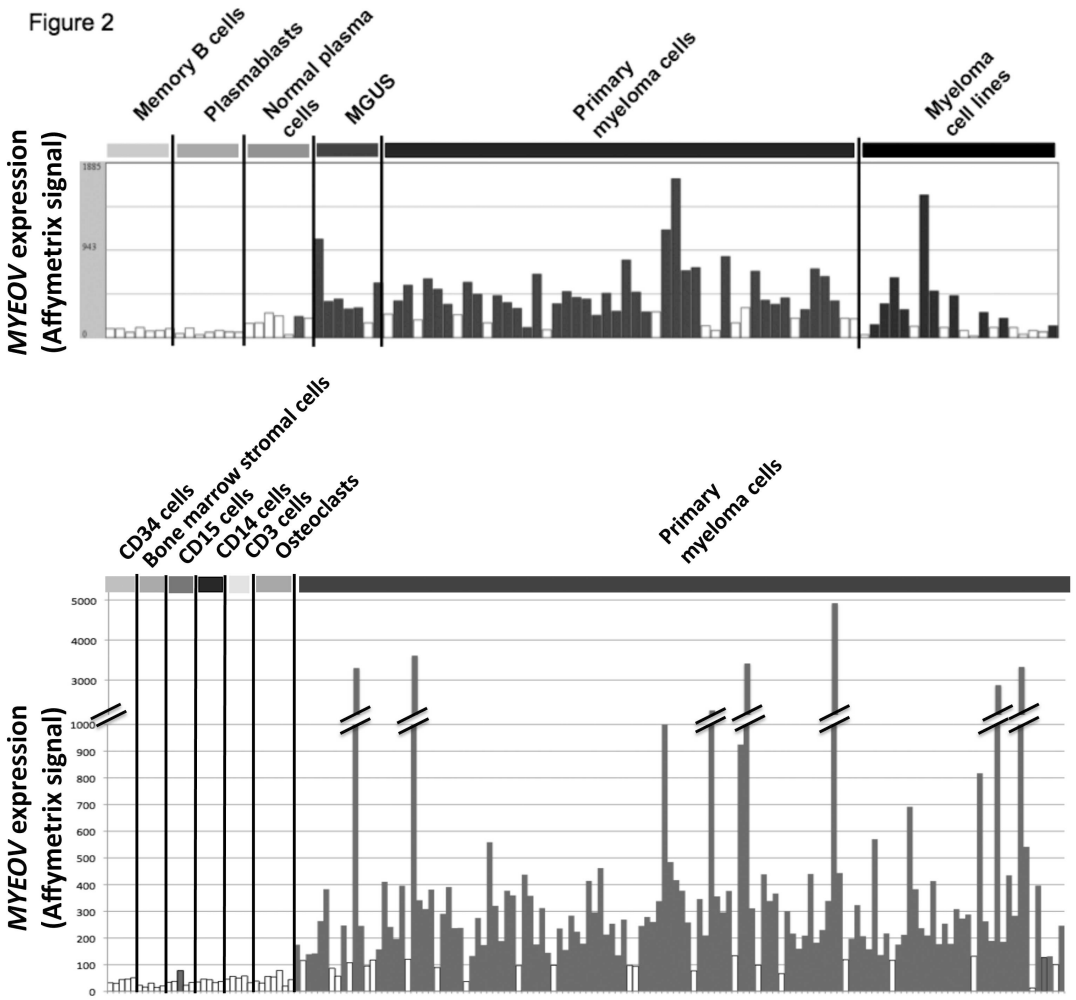
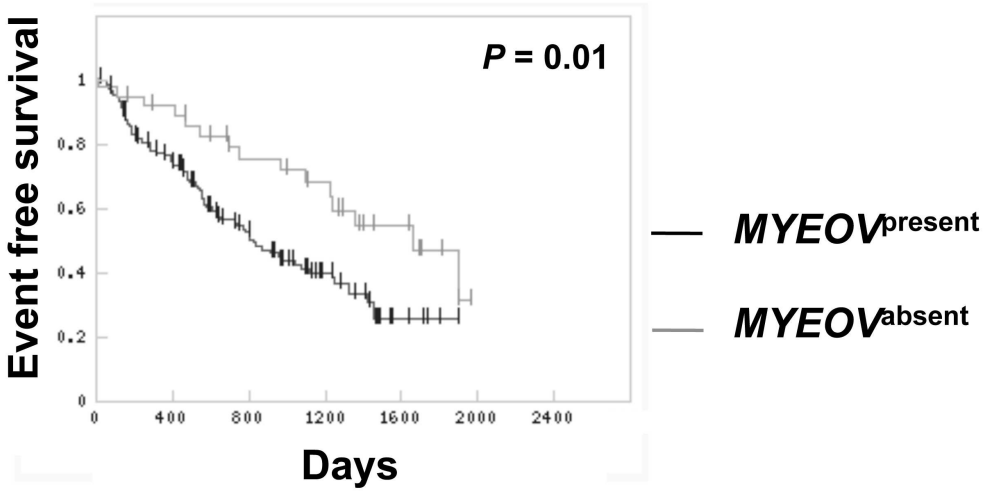
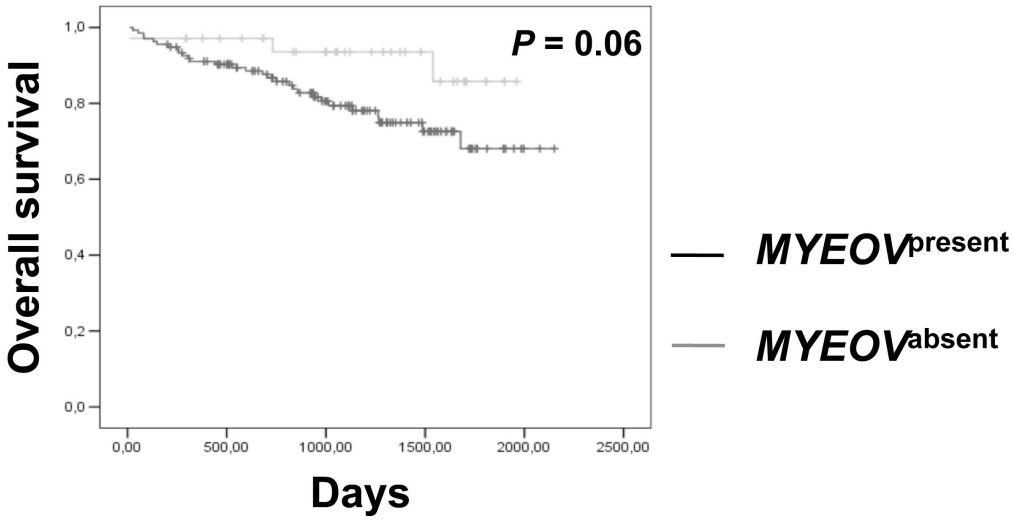

Memory B cells
Plasmablasts
Normal plasma cells
MGUS
Primary myeloma cells
Myeloma cell lines
MYEOV expression (Affymetrix signal)
1885
943
0
CD34 cells
Bone marrow stromal cells
CD15 cells
CD14 cells
CD3 cells
Osteoclasts
Primary myeloma cells
MYEOV expression (Affymetrix signal)
5000
4000
3000
1000
900
800
700
600
500
400
300
200
100
0

Figure 3 A



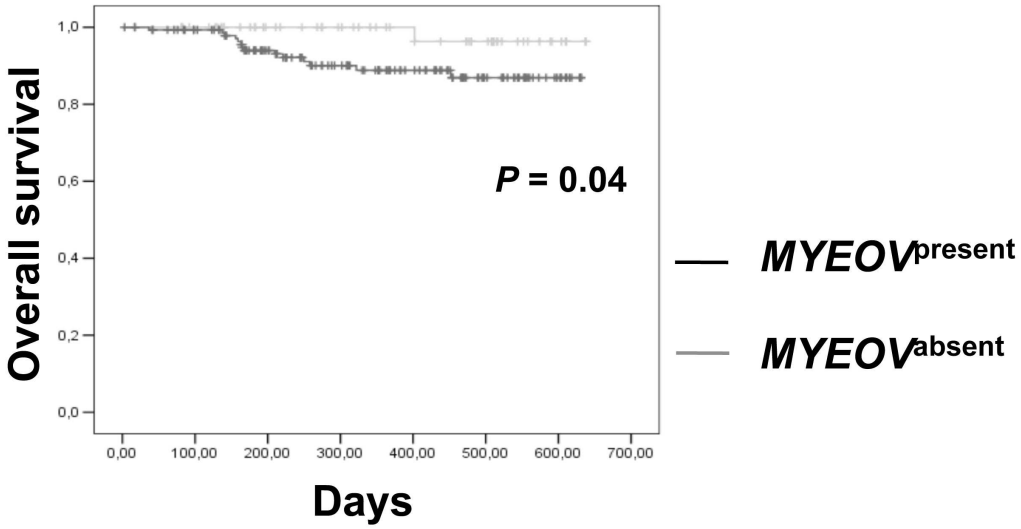
EFS HM cohort

Figure 3 B



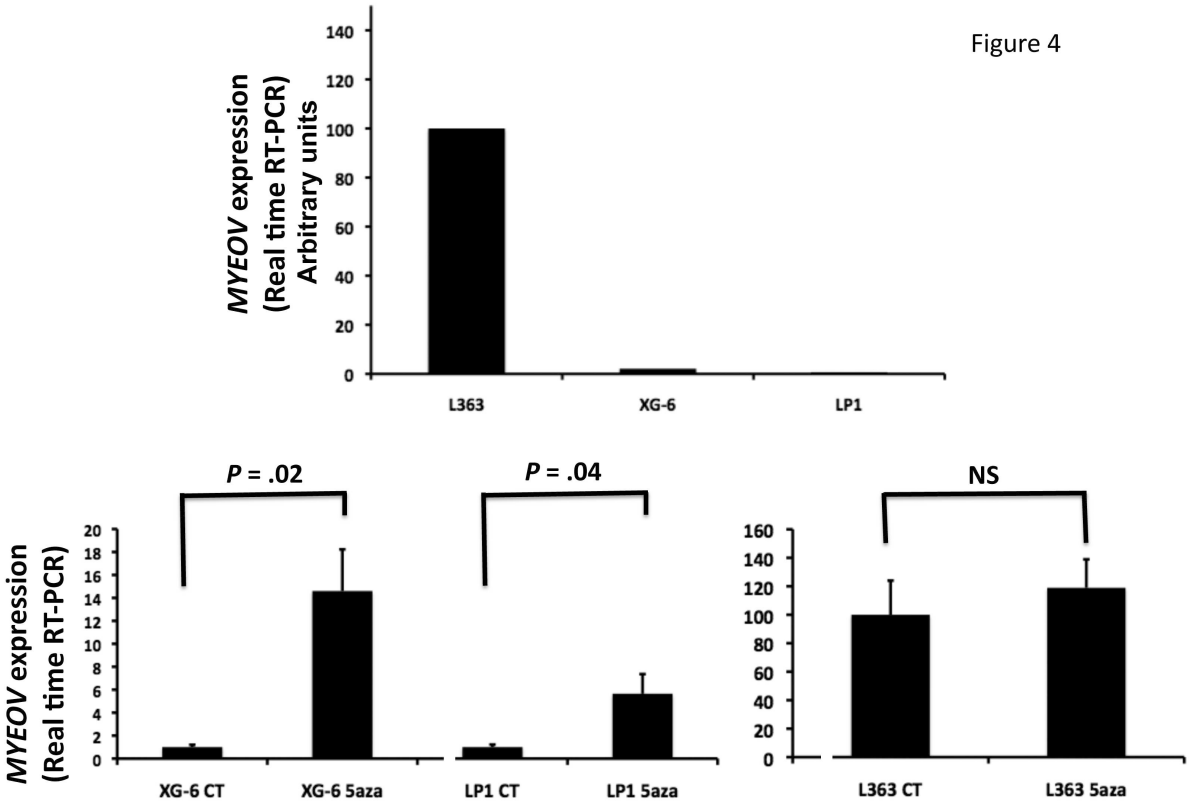
OAS HM cohort

Figure 3 C



OAS LR-TT3 cohort

Figure 4


MYEOV expression
(Real time RT-PCR)
Arbitrary units
140
120
100
80
60
40
20
0
L363
XG-6
LP1
P = .02
P = .04
NS
MYEOV expression
(Real time RT-PCR)
20
18
16
14
12
10
8
6
4
2
0
XG-6 CT
XG-6 5aza
LP1 CT
LP1 5aza
160
140
120
100
80
60
40
20
0
L363 CT
L363 5aza

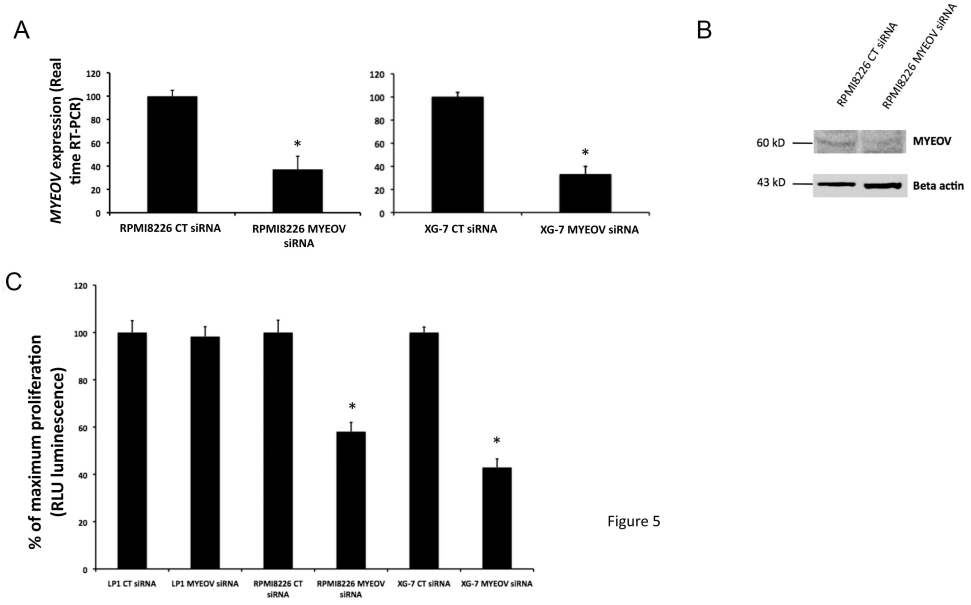
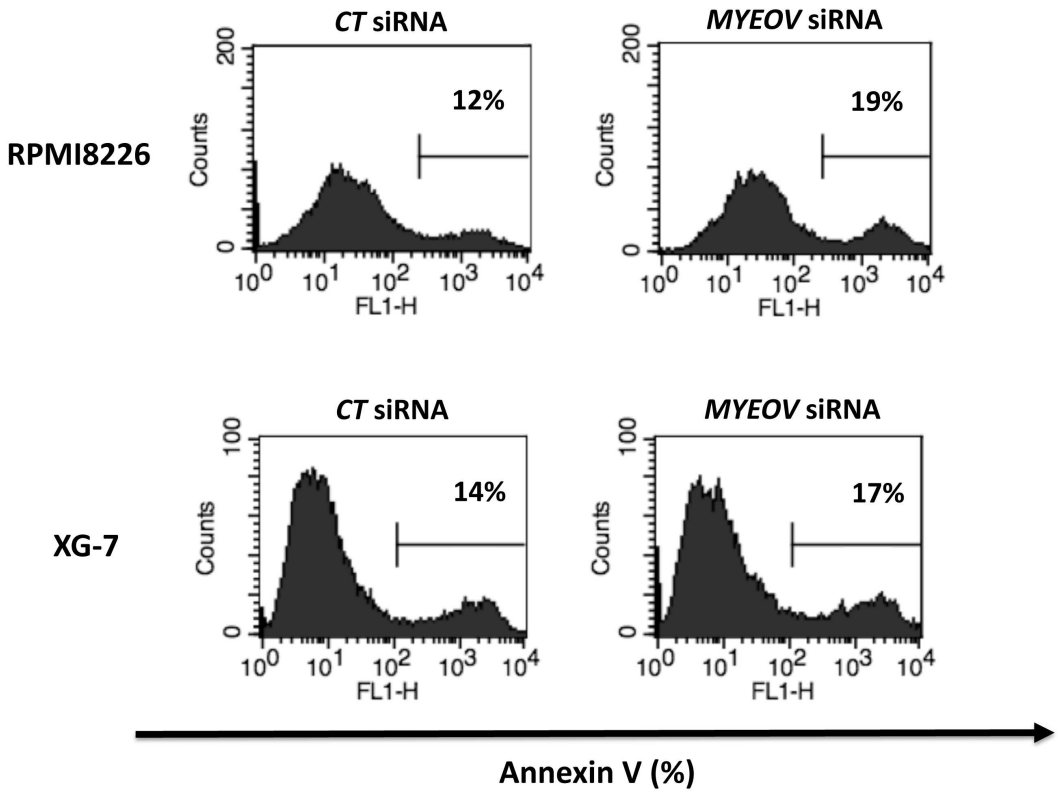

A
MYEOV expression (Real time RT-PCR)
120
100
80
60
40
20
0
*
*
RPMI8226 CT siRNA
RPMI8226 MYEOV siRNA
XG-7 CT siRNA
XG-7 MYEOV siRNA
B
RPMI8226 CT siRNA
RPMI8226 MYEOV siRNA
60 kD
MYEOV
43 kD
Beta actin
C
% of maximum proliferation (RLU luminescence)
120
100
80
60
40
20
0
*
*
LP1 CT siRNA
LP1 MYEOV siRNA
RPMI8226 CT siRNA
RPMI8226 MYEOV siRNA
XG-7 CT siRNA
XG-7 MYEOV siRNA

Figure 5

Figure 5D


RPMI8226
CT siRNA
MYEOV siRNA
12%
19%
Counts
FL1-H
XG-7
CT siRNA
MYEOV siRNA
14%
17%
Counts
FL1-H
Annexin V (%)

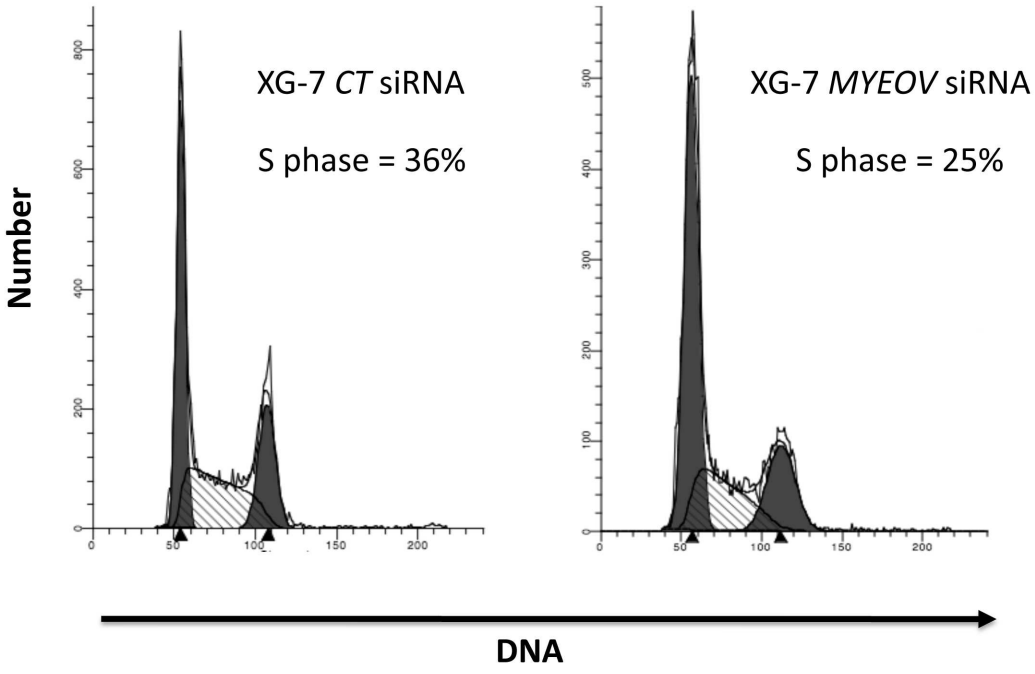


Figure 5E