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Allelic Mutations in Noncoding Genomic Sequences Construct Novel Transcription Factor Binding Sites that Promote Gene Overexpression

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Abstract

The growth and survival factor hepatocyte growth factor (HGF) is expressed at high levels in multiple myeloma (MM) cells. We report here that elevated *HGF* transcription in MM was traced to DNA mutations in the promoter alleles of *HGF*. Sequence analysis revealed a previously undiscovered single-nucleotide polymorphism (SNP) and crucial single-nucleotide variants (SNVs) in the promoters of myeloma cells that produce large amounts of HGF. The allele-specific mutations functionally reassembled wild-type sequences into the motifs that affiliate with endogenous transcription factors NFκB (nuclear factor kappa-B), MZF1 (myeloid zinc finger 1), and NRF-2 (nuclear factor erythroid 2-related factor 2). In vitro, a mutant allele that gained novel NFκB-binding sites directly responded to transcriptional signaling induced by tumor necrosis factor alpha (TNFα) to promote high levels of luciferase reporter. Given the recent discovery by genome-wide sequencing (GWS) of numerous non-coding mutations in myeloma genomes, our data provide evidence that heterogeneous SNVs in the gene regulatory regions may frequently transform wild-type alleles into novel transcription factor binding properties to aberrantly interact with dysregulated transcriptional signals in MM and other cancer cells.

INTRODUCTION

Multiple myeloma (MM) is a neoplasm of antibody-secreting plasma cells (PCs) and the second most frequently diagnosed hematopoietic malignancy (Barlogie et al., 2005). MM usually comprises several molecular subtypes with similar histological and clinical presentations (Hurt et al., 2004; Bergsagel et al., 2005; Carrasco et al., 2006; Gabrea et al.,

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2006; Zhan et al., 2006; Shaughnessy et al., 2007). Recent genome-wide sequencing (GWS) studies have revealed that MM genomes are mutated at the frequency of 2.9 per 10⁶ bases (or, 7,450 nucleotide mutations per malignant cell), and most of these mutations occur in noncoding genomic sequences that make up >98% of the human genome (Chapman et al., 2011; Weinhold et al., 2014). Other studies highlighted mutations that lead to activation of the NFκB pathway in more than a third of patients with MM (Annunziata et al., 2007; Keats et al., 2007).

In a comparative analysis of the transcriptome of bone marrow PCs from healthy donors and patients with MM, we found that hepatocyte growth factor gene (*HGF*) was the only cytokine gene significantly elevated in MM (Zhan et al., 2002). HGF regulates cell growth, cell motility, and cell adhesion by binding to the receptor tyrosine kinase Met (Kuba et al., 2000; Holt et al., 2005, 2008). A potential role for HGF in MM pathobiology has been suggested in numerous studies (Børset et al., 1996; Seidel et al., 2002; Standal et al., 2007). In association with basic fibroblast growth factor, vascular endothelial growth factor, and soluble syndecan-1, HGF significantly increases angiogenic activity within myeloma bone marrow, supporting the survival and regeneration of malignant PCs (Gherardi, 1991; Sezer et al., 2001; Andersen et al., 2005). High concentrations of HGF in MM sera are produced by myeloma cells and associated with short-term responses to therapies and early relapses of MM (Seidel et al., 1998; Rampa et al., 2014).

Given that (1) most myeloma cells significantly overexpress *HGF*, (2) healthy plasma cells do not express *HGF*, and (3) myeloma genomes are by nature heterogeneous, this study focused on the transcription regulatory region of *HGF*. On the basis of our systematic investigation, we determined that allelic DNA mutations in *HGF* promoters are frequently responsible for aberrant *HGF* overexpression in myeloma cells.

MATERIALS AND METHODS

Cell Lines and Culture

Four human myeloma cell lines, H929, OPM2, RPMI-8226, and U266, were purchased from American Type Culture Collection (Manassas, VA); two cell lines, ARK and CAG, were developed in our laboratories; seven myeloma cell lines, ANBL6, INA6, JJN3, KMS11, L363, OCI-MY1, and MM144, were kindly provided by Michael Kuehl, MD (Genetics Department, Medicine Branch, National Cancer Institute, Bethesda, MD). All cell lines were maintained in RPMI1640 medium (Lifetechnologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA), 100 U ml⁻¹ of penicillin/streptomycin, and 2 mM L-glutamine (Lifetechnologies). Cells were incubated at 37°C with 5% CO₂.

Study Subjects

To assess the newly discovered SNP in germline and myeloma PCs, bone marrow aspirates and peripheral blood stem cells (after mobilization for use in autologous transplant) from patients with newly diagnosed MM were harvested using established surgical procedures at the University of Arkansas for Medical Sciences. The study was approved by the

Institutional Review Board of the University. Informed consents were obtained and kept on record in accordance with the Declaration of Helsinki.

Nucleic Acid Preparations

Genomic DNA was isolated from myeloma cells and stem cells using the QIAamp DNA Kit (Qiagen, Valencia, CA). Normal human genomic DNA, comprising a pool of DNA from whole blood obtained from healthy male and female donors, was purchased from Clontech (Mountain View, CA). DNA from human bacterial artificial chromosome (BAC) clones was isolated and labeled as probes for fluorescent in situ hybridization (FISH). The BAC clones of RP11-133P5 corresponded to the *IL6* gene (7p15), CTD-2045M11 corresponded to the *MET* gene (7q31), and RP11-994H15 corresponded to the *HGF* gene (7q21.1). The BAC plasmids were labeled with green or red fluorochrome-conjugated dUTP using the Nick Translation Kit (Abbott Molecular, Des Plaines, IL). The pGL4.70[hRluc] (*Renilla* luciferase reporter) vector and pGL4.75 vector, which contains the hRluc reporter gene driven by a CMV promoter (pCMV), were purchased from Promega Corporation (Madison, WI).

Reverse Transcription and Quantitative PCR

Reverse transcription (RT) was initiated with 0.5 µg of total RNA and Random-Hexamer primers in the SuperScript III First-Strand cDNA Synthesis system (Lifetechnologies). An aliquot of 10 ng RNA-converted cDNA was mixed with the TaqMan Gene Expression Assays of *HGF* (Hs00300159_m1) or *GAPDH* (Hs99999905_m1) (Applied Biosystems, Foster City, CA) and amplified in the ABI 7000 real-time system (Applied Biosystems) to quantify the gene transcripts, respectively. The relative quantitation (R.Q.) of *HGF* expression levels were calculated using the comparative Ct method (2^{-Ct}). All reactions were set in triplicates.

Conventional and Molecular Cytogenetics

Procedures for metaphase chromosome preparations, FISH, and spectral karyotyping (SKY) were performed as previously described (Sawyer et al., 1998; Tian et al., 2014). The SKYPaint DNA Kit for human chromosomes was purchased from Applied Spectral Imaging (Vista, CA).

Cloning and Sequencing

HGF promoter fragments were amplified using a high fidelity PCR system (Lifetechnologies) with genomic DNA. To obtain 3.5-kb *HGF* promoter segments, 5'-TGCGTTGTGGTTTGTGGTGC-3' and 5'-AGTCAGTGCCTAAAAGAGCCAGTC-3' primers were designed for DNA amplification. The amplicons were directly sequenced or cloned into TOPO cloning vector (Lifetechnologies). Single colonies were individually sequenced using the Big-Dye Primer Cycle Sequencing Kit (Lifetechnologies) followed by electrophoresis in the 3130x Genetic Analyzer (Applied Biosystems). The 2.7-kb *HGF* promoters were subcloned into the pGL4.70 vector with modified 5' and 3' ends in an orientation of the *KpnI* site toward the *XhoI* site (New England Biolabs, Ipswich, MA). The first 47 bases of *HGF* mRNA (NM_000601) were included (Supporting Information Fig. 1).

A similar procedure was used to clone a 3-kb wild-type human *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) promoter into the pGL4.70 vector with partial sequences of *GAPDH* exon 1 and the last exon of upstream *CNAPI* gene attached.

Gene Transfection and Detection of *Renilla* Luciferase Activity

All gene transfections were carried out with 0.5 pmol of plasmid and a Lipofectamine 2000 (LF2000,) system (Lifetechnologies) at a ratio of 1:5 ($\mu\text{g}:\mu\text{l}$) per 10^6 cells in 24-well plates. To control regulatory efficiency, all gene transfections with *HGF* promoter-driven (pHGF) pGL4.70 were paralleled with *GAPDH* promoter-driven (pGAPDH) pGL4.70 and pGL4.75 vectors as positive controls and with empty pGL4.70 vector as negative control. *Renilla* luciferase activity was detected using the Dual-Luciferase Reporter Assay System (Promega) and the Victor3 1420 Multilabel Counter (Perkin Elmer Life Sciences, Boston, MA). The LF2000-assay efficiency was controlled with a pCMV-LacZ plasmid. An aliquot of transfected cells was incubated with medium containing 5 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 5 mM $\text{K}_4\text{Fe}(\text{CN})_6 \cdot 3\text{H}_2\text{O}$, 2 mM MgCl_2 , and X-Gal (1 mg ml^{-1}) at 37°C with 5% CO_2 as β -Galactosidase-positive cells gradually turned blue to indicate transfection efficiency (Supporting Information Fig. 3).

Protein Extraction for ELISA and Western Blot

Myeloma cell lines were cultured in complete medium at $10^6/\text{ml}$ and treated with recombinant human TNF α (R&D Systems, Inc., Minneapolis, MN) at 10 ng ml^{-1} . The nuclear protein fraction was isolated from 10 million cells using the Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA).

Detection of NF κ B family members in H929 cells was carried out in triplicates using 20- μg aliquot of nuclear protein per well in the TransAM ELISA Kit (Active Motif, Carlsbad, CA). All members of the NF κ B family were measured in comparison with 5 μg Raji cell nuclear protein provided by the vendor.

For Western blotting and detection of NF κ Bp50, MZF1, and NRF-2, 50 μg of nuclear proteins were separated by electrophoresis in NuPAGE 4–12% Bis-Tris gels and transferred to PVDF membranes (Lifetechnologies). Primary rabbit anti-human NF κ Bp50 antibody was obtained from Active Motif; rabbit anti-human MZF1 and NRF-2, and monoclonal antibody to human TBP were purchased from Abcam (Cambridge, MA). WesternBreeze Chemiluminescent Kits (Lifetechnologies) were used for detecting immobilized primary antibodies on the blots.

Targeted Genetic Repair of Nucleotide Mutations in the *HGF* Promoter

To rectify the point-mutations in pHGF*j03* and pHGF*j19* promoters, DpnI-mediated site-directed genetic repair was used with primer pairs that contained the wild-type sequence. New strands of the promoters were amplified using the *Pfu* DNA polymerase system (Stratagene, La Jolla, CA). The strand that contains the mutation was degraded by DpnI digestion. The nucleotide rectification was confirmed by DNA sequencing.

RESULTS

HGF Expression in MM is not Driven by Chromosome Band 7q21 Copy Number or Gross Translocations

FISH was performed on metaphase chromosomes of 12 myeloma cell lines using a probe combination of *HGF*(7q21) and *MET*(7q31) or *HGF*(7q21) and *IL6*(7p15). Eight of the 12 myeloma cell lines had more than two copies of chromosome 7, and five of these eight cell lines showed telomeric translocation and/or partial deletion of 7q. None of the tested cell lines had deletion (less than two copies) of chromosome 7 (Table 1). The levels of *HGF* expression in the myeloma cell lines were not associated with either hyperploidy of 7q or copy numbers of the *HGF* locus ($R^2=0.003$). The cell lines with the highest copy numbers of *HGF*, such as INA6 and L363, did not express higher levels of the *HGF* gene (Fig. 1 and Table 1). In contrast, of all the myeloma cell lines tested, JJN3 cells, which have only three copies of *HGF* (Figs. 1A and 1B), expressed *HGF* at the highest level.

A Novel SNP was Discovered in the HGF Promoter

DNA sequencing analysis identified a single-nucleotide polymorphism (SNP) of C to T transition at 21,485 upstream from *HGF* coding sequences (AATAGATCCCTC>AATAGATCTCTC, with the SNP underlined) of the *HGF* promoters from the myeloma cell lines. By coincidence, this polymorphism assembled an additional *Bgl*III restriction site to divide a wild-type 2.7-kb *HGF* promoter into two fragments (Fig. 2A). Overall, >80% of the promoters from the cell lines, germline, and myeloma PCs contain this SNP (Fig. 2B). The SNP did not result in gain or loss of the transcription factor binding site (TFBS) in the *HGF* promoters.

Single-Nucleotide Variants Assemble Novel Transcription Factor Binding Sites in HGF Promoters

The *HGF* promoters were amplified from the JJN3 cell line. DNA sequencing analysis revealed numerous SNVs in the amplicons. To verify these minor allele frequencies (MAFs) from the amplicons, 30 *HGF* promoter clones from JJN3 cells were individually analyzed and aligned to the human genome database. Of 30 *HGF* promoters, three isotypic alleles were identified: pHGF*j03* subtype (6/30) contains an A>G at -2,196 and a G>A at -1,477; pHGF*j04i* subtype (9/30) contains a G>A at -2,682, a SNP at -1,485, two C>T at -1,428 and -81, and an A>G at -251; and pHGF*j19* subtype (8/30) contains two C>T at -774 and -772, and two A>G at -383 at -48 (Figs. 1F and 2, and Supporting Information Fig. 1). Transcription factor binding analyses determined that pHGF*j03* gained a pair of NFkB binding sites in both DNA strands due to a G:A transition (-1,477: AGGAATTC>GGGAATTC, with the mutant nucleotide underlined); pHGF*j04i* did not gain any significant TFBS; and pHGF*j19* gained a MZF1 site and a NRF-2 site due to an A:G transition (-48: GGAGGG-GACTGGAAGAG>GGAGGGGGCTGGAA-GAG) (Fig. 2). Other SNVs at different locations were also seen in the promoters from H929 and L363 cell lines, but not associated with gain of TFBS (Supporting Information Fig. 2). The *HGF* promoters from healthy donors' DNA (purchased from Clontech) were also cloned into pGL4.70 vector and analyzed. Two types of wild-type *HGF* promoter, with or without the

SNP, were identified in the healthy donors (Fig. 2B) and used as controls compared with the *HGF* promoters from JJN3 cells in the functional assays.

The *HGF* Promoters from JJN3 Cells Are Functional

In vitro, three isotypic *HGF* promoters from the JJN3 cell line and two wild-type *HGF* promoters were validated with pGL4.70 *Renilla* luciferase reporter vector in H929, INA6, JJN3, and L363 myeloma cell lines. During 24-hr transient transfection, the pHGFj03 was highly active in driving luciferase in the INA6, JJN3, and L363 cell lines, but not in the H929 cell line. The pHGFj04i was not active in any of host cell lines. The pHGFj19 was highly active in all host cell lines. Wild-type *HGF* promoters, regardless of existence of SNP, did not exhibit regulatory activity in the host cell lines (Table 2). The pGAPDH and pCMV were highly functional in all host cell lines (Table 2).

pHGFj03 was Highly Responsive to TNF α -induced Signaling through NFKB

The pHGFj03 allele contains a G:A transition that resulted in gain of the NFKB binding site in each of the DNA strands (Fig. 2). Functionally, pHGFj03 was capable of promoting high level of reporter luciferase in the JJN3, L363, INA6 cell lines but not in the H929 cell line. Western blot assays showed high levels of nuclear NFKBp50 in JJN3, INA6, and L363 cells, with JJN3 showing the highest levels. In contrast, H929 cells had the lowest nuclear NFKBp50 (Fig. 3A). By adding TNF α to cell cultures, both nuclear NFKBp50 and NFKBp65 levels in the H929 cell line were increased (Figs. 3B and 4A). Subsequent to TNF α stimulation, luciferase activity in H929 cells transfected with pHGFj03-driven pGL4.70 vector was significantly increased (Table 1). However, the other promoters (except pCMV) did not respond to TNF α . By adding the nuclear localization signal inhibitory peptide of NFKBp50 (p50NLSB) (Lin, et al., 1995) to the culture medium, TNF α -induced luciferase activity in H929 cells transfected with pHGFj03-driven pGL4.70 vector was turned down to the baseline level (Fig. 4B). The data suggest that pHGFj03 was a highly active allele in JJN3 cells corresponding to the level of endogenous NFKB transcription factors in the myeloma cells.

The pHGFj19 allele is also an active allele in the JJN3 cell line, with an A:G transition at -48 that assembled novel MZF1 and NRF-2 binding sites (Fig. 2). The mutant pHGFj19 allele was highly responsive to high levels of MZF1 and NRF-2 in the JJN3, H929, INA6, and L363 cell lines (Fig. 3A). These data suggest that pHGFj19 was the other allele in JJN3 cells corresponding to the endogenous MZF1 and NRF-2 transcription factors in the myeloma cells.

The nucleotide mutations in pHGFj03 (G:A) and in pHGFj19 (A:G) can be repaired back to wild type under the targeted genetic rectification. Upon removal of the mutant nucleotides both pHGFj03 and pHGFj19 were no longer promoting luciferase expression in H929 cells and/or responsive to TNF α stimulation (Table 3).

DISCUSSION

The landscapes of genome-wide nucleotide mutations have been broadly investigated with a large number of biopsies from nearly 30 different types of cancers (Weinhold et al., 2014).

Prior to that work, the genomes of myeloma cells from patients who were primarily diagnosed with MM were analyzed using both whole-genome sequencing (WGS) and whole-exome sequencing (WES) approaches (Chapman et al., 2011). Mutation hotspots were detected in gene-coding sequences. Mutations in non-coding regions including introns, promoters, un-translated regions, and even intergenic sequences were also detected (Chapman et al., 2011; Weinhold et al., 2014). The WGS studies have indicated that a frequent DNA mutation at chromosome band 7q21 is a hotspot in cancer genomes (Chapman et al., 2011).

MM is a plasma cell disorder in which accumulation of molecular cytogenetic aberrations present in heterogeneous tumor subsets throughout bone marrow and focal lesions (Morgan, et al., 2012). Elevated expression of the *HGF* gene could be detected in both permanent myeloma cell lines and in PCs from patients with MM. In previous studies we and others have shown that HGF is a cytokine capable of inducing autocrine signaling to promote growth and survival of MM (Derksen, et al., 2003; Tjin, et al., 2004; Iwasaki et al., 2002; Hov, et al., 2004). Unraveling the aberrant regulatory mechanism of *HGF* overexpression in MM is important to understanding its pathogenesis and disease progression.

Initially, our cytogenetic analyses explored myeloma cells expressing high or low *HGF*. We found that copy numbers of the *HGF* locus were not the key factor associated with levels of *HGF* gene transcripts (Table 1). Next, DNA of *HGF* promoter regions from cell lines and primary myeloma plasma cells were amplified, sequenced, and aligned to the wild-type human genome. Because of the heterogeneous nature of myeloma genomes, DNA sequencing trace indicated intriguing information that led us to scrutinize into the depth of allele-specific mutations in *HGF* promoters of the JJN3 myeloma cell line, which produces the highest level of HGF amongst human myeloma cell lines and contains three copies of the *HGF* locus (Fig. 1). By carefully inspecting the cloned promoter alleles, we first discovered a novel SNP present in >80% of wild-type and MM genomes. With a C>T transition, the SNP perfectly assembled 6 nucleotides into a *Bgl*III restriction site that divides the wild-type promoter region (Fig. 2). Surprisingly, this SNP had never been reported. More importantly, our study revealed the regulatory mechanism of aberrant high expression of *HGF* in MM.

We established a model to systematically examine aberrant promoter regions in gene expression regulation. The H929 myeloma cell line that lacks *HGF* expression (Table 1) was utilized as surrogate host of the luciferase reporter vectors driven by mutant promoters from high HGF-producing JJN3 cells. Our study determined that wild-type *HGF* promoters are essentially not responsive to endogenous transcription factors (Table 2 and Fig. 3); unlike the *GAPDH* promoter that drives a housekeeping gene in an array of healthy and tumor cell types. In contrast, the mutant promoters, i.e., pHGFj03 and pHGFj19 alleles, from JJN3 cells were highly functional. In vitro, these mutant alleles actively promoted luciferase expression not only in the JJN3 cell line but also in myeloma cell lines that do not express *HGF* but have high levels of endogenous or inducible transcription factors binding to the novel TFBS (Table 2 and Fig. 3). We also demonstrated that the H929 cell line could be an ideal research tool directly responding to TNF α induced signaling pathway to express high levels of NFkB (Fig. 3) that activated the mutant *HGF* promoter (pHGFj03) to upregulate luciferase in the host cells. Moreover, our study determined that the rectification of allele-

specific mutations in *pHGFj03* and *pHGFj19* has transformed DNA sequences into wild-type sequences that were no longer active in the host myeloma cell line. These functional assays suggest that allele-specific mutations in the promoter regions of proto-oncogenes are crucial to overexpression of the genes in cancer cells.

By using heterogeneous genomic DNA templates from cell lines to study non-coding sequences of myeloma genomes, we demonstrated that allele-specific mutations may be the dominant factor in transforming wild-type non-coding genomic sequences of the promoter region of proto-oncogenes such as *HGF* (typically not expressed in healthy cells). The mutant sequences with novel transcription factor binding properties may become highly responsive promoters corresponding to dysregulated signal transduction in cancer cells, e.g. the H929 cells. Despite the remarkable progress made through next-generation sequencing technologies, our study showed an overwhelming amount of MAFs present in each case of abnormal oncogene overexpression, suggesting that the ultimate solution to understanding the regulatory mechanism of aberrant gene transcriptions requires more sophisticated and systematic analyses to identify key factors within each allele of a cancer genome.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Karyotypic analyses and FISH of myeloma cell lines. **A.** SKY of a JJN3 metaphase. **B.** SKY analysis of JJN3 cells. Chromosomes, differentiated by colors, are arranged in numeric order by the computer software. **C.** JJN3 metaphase chromosomes were hybridized with *HGF* (7q21 in green) and *MET* (7q31 in red) probes. Note three copies of *HGF* (in green). **D, E.** INA6 (left) and L363 (right) metaphase chromosomes were hybridized with *HGF* (7q21 in red) and *IL6* (7p15 in green) probes. INA6 cells show four copies of chromosome 7 (ch7) and L363 have six copies of the chromosome. HGF: the additional genomic material from

chromosome arm 7q containing the *HGF* locus on a derivative chromosome. **F** The chromatographs of DNA sequencing trace of heterogeneous genomic templates from JJN3 cell lines. The double peaks (▲) indicate SNP or single nucleotide variant (SNV).

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Figure 2.

SNP and heterogeneous nucleotide mutations in the promoter alleles of HGF in JJN3 myeloma cell line. **A:** ① A novel SNP is located at –1,485 from the exon 1. The C>T transition forms an additional Bgl II site in 2.7 kb promoter region. ② A single nucleotide variant (A>G) at –1 477 assembles wild-type sequences into NFKB binding sites in pHGFj03. ③ + ① A T>C transition at –81 co-exists with a SNP in pHGFj04i. ④ A single nucleotide variant at –48 assembles wild-type sequences into MZF1 and NRF2 binding sites in pHGFj19. **B:** The HGF promoter regions were amplified from myeloma cell lines, healthy donors (Normal), and patients diagnosed with MM. The PCR products were digested with the restriction endonuclease BglII to determine the existence of a novel SNP that truncates the promoter into 1,504 bp and 1,197 bp fragments. pc: CD1381- selected myeloma cells. st: peripheral blood stem cells; #: none of the alleles has the SNP; *: all alleles have the SNP. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Figure 3.

Western blot and chemiluminescent immune-detection. **A.** The nuclear proteins (50 µg) from H929, INA6, JN3, and L363 cell lines were analyzed using the Western blot protocol for NFKBp50, MZF1, NRF2, and TATA box binding protein (TBP). **B.** The comparison of nuclear NFKBp50 from H929 (lane 1–4) and JN3 cell lines (lane 5– 6) after the cells were treated with (+) or without (–) TNFα (10 ng ml⁻¹) for 6 or 24 hours (hr). 50µg of nuclear proteins were loaded in each lane aside with 5µg of positive control nuclear proteins from RAJI cell line (lane 7). TBP: the loading control. Numbers (at left) indicate molecular weight (kilodalton).

Figure 4.

A. H929 myeloma cells were incubated with the complete medium contains TNFα (10 ng ml⁻¹) for 6 and 24 h. The nuclear protein fractions (20 μg per well) were analyzed using TransAM ELISA kit for the quantity of the members of NFκB family comparing to the nuclear proteins of Raji cell and the negative control (blank), in triplicate. Bars are standard deviations of the triple measurements. **B.** H929 cells were transiently transfected with pHGF^{wt}- and pHGF^{j03}-driven luciferase reporter vectors and incubated in the complete medium with or without TNFα (10 ng ml⁻¹); 100 μM nuclear location signal-blocking agent (p50NLSB) was added to the cells prior to TNFα stimulation. Luciferase activity was measured at 24 hrs. Each column represents the mean of three parallel measurements; bars indicate standard deviations. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 1

HGF Gene Expression Quantitation and *HGF* and *Met* Gene Copies by FISH in the Human Myeloma Cell Lines

Myeloma cell lines	qPCR R.Q. ^a	Copies of <i>HGF</i> / <i>Met</i> ^b
ARK	0.0	3/3
H929	0.0	2/2
IMA6	0.0	6/6
L363	0.0	6/6
OPM2	10.3	2/4
KMS11	14.0	3/4
U266	83.9	2/3
RPMI8226	140.6	3/3
MM-144	929.3	2/2
OCI-MY1	5,311.9	3/3
ANBL6	14,066.7	5/6
JJN3	33,110.5	3/2

^a *HGF* gene transcripts were quantitatively measured using RT-*TaqMan* PCR. The relative quantitation (R.Q.) was calculated using the comparative C_t method (2^{-C_t}) based on the negative cell lines of ARK, H929, IMA6, and L363.

^b FISH (fluorescence in situ hybridization) with *HGF* and *Met* probes performed on metaphase chromosomes of the myeloma cell lines. The copy numbers of each probe were scored from 20 metaphases for each cell line.

TABLE 2

Luciferase Activity in the Myeloma Cell Lines 24 Hrs Post Transfection of pGL4.70 [*hRluc*] Reporter Vector Driven by Various *HGF* Promoters and the Assay Control Promoters

	H929	INA6	JJN3	L363	H929/TNFα^a
<i>pHGFβ03</i>	575	2,157	116,897	453,751	4,046
<i>pHGFβ04i</i>	1,558	1,251	87,338	194,306	1,753
<i>pHGFβ19</i>	2,526	2,978	101,636	448,854	2,847
<i>pHGFwt</i>	564	299	2,119	14,027	681
<i>pHGFwti</i>	272	352	2,039	25,856	556
<i>pGL4.70Opmt</i>	245	697	1,585	6,301	465
<i>pGAPDH 3kb</i>	60,676	322,868	16,902,973	15,499,502	84,647
<i>pGL4.75 (CMV)</i>	5,702,975	8,156,375	510,418,512	191,166,656	13,294,915

The promoter sequences contain the SNP.

^a H929 cells were cultured in complete medium supplemented with 10 ng ml⁻¹ TNF α .

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TABLE 3

Luciferase Reporter Activity in H929 Myeloma Cell Line 24 Hrs Post the Transient Transfection with HGF Promoters Cloned from the JLN3 Cells in pGL4.70 [*hRluc*] Vector and TNF α Stimulation

Plasmids	TGR ^b	Non-stimulus (24 h)		With TNF α ^a (24 h)	
		Mean	SD	Mean	SD
<i>pHGF.j03</i>	Ø	503	±26	4,068	±312
<i>pHGF.j03</i>	G>A (-1477)	472	±48	318	±5
<i>pHGF.j19</i>	Ø	2,550	±112	1,403	±80
<i>pHGF.j19</i>	A>G (-48)	575	±29	651	±41

^aH929 cells were transiently transfected with eight recombinant reporter plasmids and incubated in complete RPMI 1640 medium supplied with 10 ng mL⁻¹ TNF α .

^bThe mutations in pHGF/*j03* and pHGF/*j19* were rectified by targeted gene repair (TGR).