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Neurobeachin (*NBEA*) is a target of recurrent interstitial deletions at 13q13 in patients with MGUS and multiple myeloma

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Abstract

Objective—Chromosome 13 deletions (del[13]), detected by metaphase cytogenetics, predict poor outcome in multiple myeloma (MM), but the gene(s) responsible have not been conclusively identified. We sought to identify tumor suppressor genes on chromosome 13 using a novel array comparative genomic hybridization (aCGH) strategy.

Methods—We identified DNA copy number losses on chromosome 13 using genomic DNA isolated from CD138 enriched bone marrow cells (tumor) from twenty patients with MM, monoclonal gammopathy of undetermined significance (MGUS) or amyloidosis. We used matched skin biopsy (germline) genomic DNA to control for copy number polymorphisms and a novel aCGH array dedicated to chromosome 13 to map somatic DNA gains and losses at ultra-high resolution (>385,000 probes; median probe spacing 60bp). We analyzed microarray expression data from an additional 262 patient samples both with and without del[13].

Results—Two distinct minimally deleted regions at 13q14 and 13q13 were defined that affected the *RBI* and *NBEA* genes, respectively. *RBI* is a canonical tumor suppressor previously implicated in MM. *NBEA* is implicated in membrane trafficking in neurons, PKA binding, and has no known role in cancer. Non-coding RNAs on chromosome 13 were not affected by interstitial deletions. Both the *RBI* and *NBEA* genes were deleted in 40% of cases (8/20; 5 patients with del[13] detected by traditional methods and three patients with interstitial deletions detected by aCGH). Forty-one additional MM patient samples were used for complete exonic sequencing of *RBI*, but no somatic mutations were found. Along with *RBI*, *NBEA* gene expression was significantly reduced in cases with del[13].

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Conclusions—The *NBEA* gene at 13q13, and its expression, are frequently disrupted in MM. Additional studies are warranted to evaluate the role of *NBEA* as a novel candidate tumor suppressor gene.

Keywords

Multiple Myeloma; array comparative genomic hybridization; *RBI*; *NBEA*

INTRODUCTION

Numeric or structural chromosomal abnormalities are detected in nearly all patients with plasma cell dyscrasias, including primary amyloidosis, monoclonal gammopathy of undetermined significance (MGUS) and multiple myeloma (MM) [1]. Chromosome 13 deletions, most frequently monosomy 13 (del[13]) are detected in 10–20% of MM cases by routine cytogenetics or metaphase fluorescent in situ hybridization (FISH) and are a significant predictor of shortened survival [2],[3], and [4]. Previous efforts to map somatically acquired DNA copy number losses on chromosome 13 in MM have identified 13q14-q21 and 13q34 as commonly affected regions [5], [6], [7], [8], [9], [10], and [11]. These studies used 10Mb resolution CGH and 1Mb resolution FISH techniques. Whole genome array CGH (0.73Mb resolution) combined with gene expression analysis identified *CUL4A* (13q34) as a potentially relevant gene located within a 0.77Mb deletion on chromosome 13 [12] and [13]. Single nucleotide polymorphism analysis (10Kb resolution) revealed a 1.9Mb minimally deleted region (MDR) spanning 13q13.3 to q21.3 [14]. The relatively low resolution of these studies has precluded consistent identification of biologically relevant genes targeted by del[13]. Additional studies using higher resolution techniques therefore, are needed.

The widespread contribution of DNA copy number polymorphisms to variability among human genomes has recently been appreciated [15] and [16]. In previous CGH studies that have used pooled genomic DNA as controls, false positive identification of DNA gains and losses due to DNA copy number polymorphisms could not be excluded. Here, we definitively identified somatic changes by using genomic DNA isolated from matched skin biopsy specimens from our patients to control for DNA copy number polymorphisms. Furthermore, we used a novel, chromosome 13-dedicated CGH array to identify genes affected by DNA copy number loss with unprecedented resolution. Our novel approach allowed the mapping of extremely small deletions. We found a 3.49Kb MDR in 13q14 that mapped to exon 20 of *RBI* encoding the highly conserved ‘pocket domain’ responsible for binding E2F transcription factors [17], [18], and [19]. A second MDR at 13q13 was definitively mapped to *NBEA*, a gene encoding a PKA binding site and predicted to regulate membrane transport in neurons with a role in cancer that has yet to be defined.

MATERIALS AND METHODS

Patient samples

Bone marrow and skin biopsy samples were obtained from patients with plasma cell dyscrasias following informed consent. The study was approved by the Washington University Institutional Review Board and the Siteman Cancer Center Patient Review Monitoring Committee. Clinical data including routine cytogenetics and M-FISH were obtained anonymously using Unique Patient Numbers (UPN). I-FISH was performed on marrow sections using probes: LSI 13/*RBI*, and *CEP7* (Vysis Inc., Downers Grove, IL, USA). For each hybridization, a minimum of 100 non-overlapping nuclei were analyzed. For molecular analyses, CD138+ bone marrow mononuclear cells were isolated by Ficoll gradient (Stem Cell Technologies, Vancouver, BC) followed by separation using CD138 microbeads and an AutoMACS Cell Separator (Miltenyi Biotec, Auburn, CA). Fluorescence activated cell-sorting

analysis using a PE-CD138 antibody (Miltenyi Biotec, Auburn, CA) confirmed >97% purity. Genomic DNA was isolated with Qiagen Miniprep Kits (Valencia, CA).

Array CGH Platform and analysis

The first 20 patient samples with $\geq 500,000$ CD138+ cells were selected for aCGH, which required 1.5 μ g DNA. CD138+ (tumor) DNA was labeled with Cy3 and skin (germline) DNA was labeled with Cy5. The custom array contained 385,272 oligonucleotide probes. Nimblegen built the array, performed probe design, and sample hybridization to the custom array (www.Nimblegen.com). Sequence source for the probe design was HG17/UCSC (<http://genome.ucsc.edu/>).

Circular Binary Segment (CBS) Analysis: CBS [20] was performed using Signal Map Software (Nimblegen, Madison, WI). Data was analyzed using a non-overlapping window, which averaged the signal intensity from each probe over a 600bp region. Since probes were spaced approximately every 60bp apart, each window averaged 10 probes. This approach was used to condense the data and provided clean segment breaks. Systematic criteria set to eliminate false positives included: ≥ 3 data points involved (representing ~ 30 probes; 180bp) and $\log_2$ ratio < -0.25 . Magnified plots were generated with Graphpad/Prism 4, Version 4.02 (Graphpad Software Inc, San Diego, CA).

Process Control analysis

Process Control analysis was performed on unaveraged data set (no windows used to condense data). Data was normalized using qspline [21]. To eliminate outliers, the raw data for the skin (reference) samples from each patient was analyzed. Probes with signal intensity $> 3X$ SD above the mean were discarded (range: 8,000–17,000, averaged 10,000 per patient (2–4.4% of total)). Process control employs techniques using a Shewhart control chart [22], a graphical and analytical tool used in industry for quality control purposes. It is applied to aCGH analysis to determine which probe intensities are different enough from mean variability to be considered meaningful. Probe intensity ratios were considered “significant” if they satisfied: eight probes *in a row* on one side of the overall mean. They also had to pass *either* A) two of three probes in a row beyond two units of overall SD, *or* B) four of five points in a row beyond one unit SD [23,24]. If an eight-probe region (representing ~ 480 bp) passed the criteria, the first and last of the eight probes were mapped. Genes were mapped by aligning probes of interest to the Human Build 36.2 genome. Whole chromosome plots using this same data set were generated using the program [R] (Figure 1A).

PCR and sequence analysis of *RB1*

High-throughput sequence analysis of *RB1* was performed by the Genome Sequencing Center at Washington University (WUSTL) as described [25]. Detailed protocols are available on WUSM GSC website (<http://genome.wustl.edu/platforms.cgi?id=7>).

PCR validation of *RB1* micro-deletion was performed on genomic DNA isolated from CD138 + selected bone marrow (tumor) and skin biopsy (germline) patient samples. The independent control DNA was kindly provided by Rhonda Reis, Division of Oncology, WUSTL. Primers: RBValFWD3: CCATTGCCCCACAGTCAGAAA
RBValREV3:GGTAGGGGAATAGGGGGTGA. Products were cloned into and sequenced from TOPO2.1 vector (Invitrogen, Carlsbad, CA).

Real Time PCR validation

Assays were performed on original patient genomic DNA with Taqman Universal PCR Master Mix. Primer concentration: 900nM; probe concentration: 2.5mM, 10ng template. Reactions

were run on 7300 Real Time PCR System, and analyzed using 7300 System Software (Applied Biosystems, Foster City, CA). RBRTFwd1:5'
GAATTAGAACATATCATCTGGACCCTTT3'

RBRTRev1:5'GGTCCAAATGCCTGTCTCTCA3'

RBExon20Probe:5'56FAMCCAGCACACCCTGCAGAATGAGTATGAA36-TAMSp3'

Glypican6Fwd:5'TTCTGGTTTCGGGCAAACCTTG3'

Glypican6Rev:5'GAAGGCGCCACTCAGACTGT3'

Glypican6Probe:5'56FAMCGACCGCAGTTTGGCCAGCG36-TAMSp3'

NBEARTF1:5'AATGGGTTACTACTGAAAACCTAGTGTA3'

NBEARTR1:5'TCGCCATCTAGTTTCATCAGTATACAG3'

NBEAProbe:5'56FAMCACAGAAAACCTGAAATTGGGAGGCTTATGTGTAA36-TAMSp3'

The $\Delta\Delta C_t$ method was used since equal efficiency of primer/probes was shown.

Microarray Expression Analysis

Two datasets were analyzed. The Mayo Clinic dataset included 162 samples [26](101 MM, 24 SMM, 22 MGUS, and 15 normal PC's; GEO GSE6477). Chromosome 13 status was determined by FISH. The MMRC dataset (<http://www.themmr.org>) included 100 samples. Chromosome 13 status was determined by aCGH. Expression values were derived against a PM/MM difference background using Robust Multichip Average (RMA, [27]). Present/Absent probes were called using Affymetrix Microarray Suite version 5. Only probes detected in at least one sample were used in subsequent comparisons. In pooled Chromosome 13 Deletion vs. No Deletion comparisons, Significance Analysis of Microarrays (SAM, [28]) was used to detect differentially expressed genes based on a q-value of less than 5%. SAM was run with 100 permutations for correction of False Discovery Rate. These genes were clustered and visualized in DChip [29] (<http://www.dchip.org>). aCGH data was first smoothed with region=2, outlier scale =4, smoothing SD=2 and trimming proportion of 0.025. CBS was then run with default parameters (alpha=0.01, window.size= NULL, with 10000 permutations).

Q-RT PCR

RNA was isolated using Trizol and cDNA generated using SuperScript First Strand Synthesis Kit (Invitrogen, Carlsbad, CA) per manufacturer's directions.

NBEAExon49F:ACTACTTGACTTATGAAGGCTCTGTGAA

NBEAExon50Rev:TGGCGTCTGTCCAAAGTTCTG

NBEAProbe:5'56FAMCAGGGAGGCCATGGAGGCACAGTAMSp3'

hGAPDHFwd:GAAGGTGAAGGTCGGAGTC

hGAPDHRev:GAAGATGGTGATGGGATTTTC GAPDHPProbe:5'

56FAMGGCTGAGAACGGGAAGCTTGATAMSp3' Human brain sample provided by Bob Schmidt, Pathology, WUSTL. Samples were run in triplicate and performed twice. Error bars are SD of two experiments.

Western blot and cell lines

LP-1, KMS-11, OPM-2 and UTM2 lines were provided by W. Michael Kuehl, Genetics Branch, NIH) and maintained in RPMI with 1% Penicillin/Streptomycin (both Cambrex Bioscience, Walkersville, MD), 10% fetal bovine serum (HyClone, Logan, Utah). RPMI-8226, U266, and H929 cells were obtained from and grown per ATCC recommendations. Lysates were prepared as described [30]. Antibodies: total RB1: IF8 (Santa Cruz Biotechnologies, Santa Cruz, CA); anti-phospho-RB1(Serine Ser807/811; Cell Signaling, Beverly, MA); Actin (Sigma, St. Louis, MO). NBEA antibody kindly provided by Manfred Kilimann, Institute for Anatomy, Bochum, Germany and used as described [40].

RESULTS

Patient characteristics

Deletions affecting chromosome 13 occur at similar frequencies in a variety of plasma cell dyscrasias [31], so patients with the diagnosis of MM, MGUS, or amyloidosis, regardless of chromosome 13 status, were selected for array comparative genomic hybridization (aCGH). Twenty patients were selected solely on the basis bone marrow plasma cell yield after CD138 enrichment (Table 1 and S1). We excluded low-yield samples to avoid the need for whole genome amplification (WGA), which can introduce bias or mutation (personal communication Matthew Walter, 2008). Genomic DNA was isolated from CD138-enriched bone marrow samples (tumor) as well as from patient-matched skin biopsy samples (germline) controls. Patient-matched skin biopsy samples were an important internal control for copy number polymorphisms known to occur in healthy populations [15] and [16]. To identify DNA copy number alterations across chromosome 13 with the greatest possible resolution, we performed comparative genomic hybridization using a custom CGH array (Nimblegen Inc, Madison, WI) dedicated to chromosome 13. The custom array had 385,272 probes spanning the entire length of chromosome 13 with median probe spacing of 60 base pairs.

DNA copy number losses identified by circular binary segment analysis

Array CGH data were plotted linearly along chromosome 13 using $\log_2$ tumor: germline signal intensity ratios (Figure 1). By eye, some regions of copy number change were obvious, but to systematically identify regions of DNA copy number loss across chromosome 13, we performed two independent, unsupervised analyses of the data. To facilitate identification of minimally deleted regions (MDRs) across patient samples, we first used a CBS algorithm using stringent criteria to identify interstitial deletions [20]. By CBS analysis, eight of the 20 patient samples (40%) harbored at least one region of interstitial DNA copy number loss with a mean deletion size of 596Kb (range: 1.2Kb to 16Mb, Table 2). Among the eight patients with DNA copy number loss, the mean number of deletions was five (range: 1 to 13). The finding of a greater number of chromosome 13 deletions than previously reported using lower resolution techniques [32] suggested that our strategy could be useful for finding novel regions on chromosome 13 contributing to plasma cell diseases.

Array CGH identifies interstitial deletions not detected by FISH or cytogenetics

We compared chromosome 13 status determined by aCGH to analyses of chromosome 13 using standard techniques including metaphase cytogenetics, metaphase fluorescent in situ hybridization (M-FISH) and interphase fluorescent in situ hybridization (I-FISH; Figure 1). Because the aCGH raw data were normalized to balance fluorochrome intensity, monosomy 13 (i.e. non-interstitial deletions) was undetectable via aCGH analysis. We were therefore forced to rely on clinical cytogenetic data for detection on monosomy 13 (Table 1, Figure 1). By cytogenetics, five of the 20 patient samples (25%) had monosomy 13 (Table 1, Figure 1). Additionally, two patients with monosomy 13 (22848 and 92896) also had aCGH-detected

DNA copy number losses suggesting homozygous deletion at those loci (Table 2). A 1200bp deletion in patient sample 22848 did not map to known genes or micro RNA at 13q31, while patient sample 92896 harbored two deletions affecting *KATNAL1* and *DNAJC3* genes.

Notably, cytogenetic and FISH analysis failed to detect chromosome 13 DNA copy number loss in 25% of cases (5/20) that were positive by aCGH (Figure 1). This data demonstrates that high-resolution array CGH has the ability to detect chromosome 13 deletions undetected by standard FISH and cytogenetics. This result also highlights the utility of unbiased analysis of the entire chromosome to identify novel regions on chromosome 13 whose copy number changes could direct the study of genes relevant to MGUS and MM pathogenesis.

Mapping of chromosome 13 genes affected by DNA copy losses

To identify chromosome 13 genes whose loss could contribute to MM pathogenesis, we mapped all known genes that fell within the regions of copy number loss identified by CBS analysis. We found 28 of 43 (65%) deleted segments mapped to at least one gene, rather than non-coding DNA (Table 2). None of the regions identified in our study mapped to known non-coding RNA loci. Specifically, the two micro RNA clusters on chromosome 13 known to contribute to CLL (miR 15–16 at 13q14 and MiR 17–92 at 13q31.3) were not affected in our samples.

To independently identify genes with copy number loss on chromosome 13, we performed a separate, unsupervised analysis of the data by using an independent Process Control algorithm [22] shown to reliably call aCGH probe signals that deviate significantly from baseline (Figure S1). This second analysis identified 216 probes that mapped to 42 genes (Table S2). Twenty of the 42 genes (48%) identified by Process Control were also identified by the CBS analysis, underscoring the robustness of the aCGH data set (Table 2).

RB1 and *NBEA* are recurrent targets of interstitial deletions in MGUS and MM

The region most affected in our patient group encompassed 13q12 to 13q14.3, (25 to 50Mb), Figure 1). CBS analysis of the log₂ plots from three of five patient samples with interstitial deletions (58762, 64511, and 95295) revealed two distinct MDRs within 13q12–14.3 (Figure 1 shaded bars, Figure 2–3). Patient sample 95295 harbored two DNA copy number losses that were extremely small (106Kb and 1200bp, respectively) and defined the MDRs at 13q14.2 and 13q13 (Figure 1–3, Table 2). Within 13q14.2, only the *RB1* gene was affected in all three patient samples (Figure 1, 2). Strikingly, the 13q14.2 MDR mapped to exon 20 of *RB1*, encoding the ‘pocket domain’ of RB1 critical to its tumor suppressor function [33].

Inspection of the log₂ plots from the same three patient samples revealed each harbored a second and distinct interstitial deletion at 13q13, 13Mb centromeric to the *RB1* locus (Figure 1, 3). This second 13q13 MDR mapped to a single gene not previously implicated in myeloma biology: neurobeachin (*NBEA*, *BCL8B*, Figure 3). Every patient sample in our set that harbored a deletion affecting *RB1* (three with interstitial deletions and five with monosomy 13) simultaneously harbored copy number losses affecting the novel myeloma associated gene *NBEA* (Table 2, Figure 1–3).

Confirmation of interstitial deletions affecting *RB1* and *NBEA* genes

Since the segment of DNA copy number decrease within *RB1* in patient 95295 was small (3.49Kb) and contributed significantly to the mapping of the 13q14 MDR, we first performed PCR spanning the microdeletion on the same tumor and skin genomic DNA used in the aCGH analysis (Figure 2). Amplification of a truncated band and sequence analysis of the PCR product confirmed this deletion tumor-associated (Figure 2). This result confirms the micro-deletion affecting *RB1* in patient sample 95295.

To quantify and confirm the DNA copy loss across all three patient samples with interstitial *RB1* deletions (58762, 64511 and 95295), real time PCR was performed on CD138 purified tumor genomic DNA (Figure 2). Consistent with the qualitative PCR, patient sample 95295 had virtually no signal using a primer-probe set at this locus (fold copy number: 0.02). Patient samples 58762 and 64511 had a fold copy number of 0.86 and 0.62, respectively, consistent with loss of one copy of *RB1*. These results are concordant with the aCGH log₂ ratios for this region (average log₂ ratio of probes that span microdeletion: 95295: -0.977; 58762: -0.518; 64511: -0.754).

A similar analysis was used to quantify and confirm the interstitial *NBEA* deletions in patient samples 58762, 64511, and 95295. Consistent with the aCGH data, patient sample 95295 revealed homozygous deletion (fold copy number: 0.14). Patient samples 58762 and 64511 revealed heterozygous loss of *NBEA* (fold copy number: 1.22 and 0.9, respectively; Figure 3). These data confirm non-contiguous interstitial deletions on chromosome 13, affecting simultaneously the *NBEA* and *RB1* genes in three of 20 patient samples (15%).

Un-mutated RB1 protein remains expressed in MM patients

Our data suggested *RB1* is a target of deletions in MM, yet in most patient samples (7/8 in our set) the other copy is retained. Limited sequence analysis in myeloma failed to show mutations in *RB1* exons 20–24 [34] (mutation hotspots in retinoblastoma) [35], but other domains of *RB1* have not been re-sequenced in MM. We performed sequencing of all 27 *RB1* exons and surrounding intronic sequences in 41 MM/MGUS patient samples (including 16 of our 20 patient set; Table S3). We found no non-synonymous sequence changes affecting the coding or promoter sequences (bp -474 to -182) of *RB1*, suggesting that, in contrast to retinoblastoma tumors, most myeloma tumors retain at least one wild-type *RB1* allele.

We detected eleven intronic SNPs (Table S3). Since myeloma is twice as prevalent in African American populations compared to Caucasians (www.seer.cancer.gov), race matched minor allele frequencies (MAFs) from our patient samples were compared to published MAFs in the Hap Map database for the nine of eleven SNPs with available data (Table S4). Two *RB1* SNPs (rs198580 and rs198617) were significantly more common in our Caucasian patients ($P < 0.001$ and $P < 0.018$, respectively), suggesting a possible role in MM pathogenesis. No significant differences were found between subgroups for the other seven SNPs.

We identified six novel SNPs, not reported in the NCBI or HapMap databases (Table S5). These were validated by repeat sequencing and were detected in both tumor and matched skin genomic DNA, demonstrating these to be germline sequence variants. Since identified SNPs were located near exon boundaries (range: 10–171bp from boundary), we considered the possibility that *RB1* SNPs might play a role in MM pathogenesis by affecting RNA splicing. Examination of *RB1* cDNA isolated from seven patients with reported or novel SNPs revealed only *RB1* transcripts of expected size (not shown). Together, our re-sequencing analysis demonstrated no somatic mutations in retained *RB1* alleles.

To determine whether retained *RB1* alleles were expressed, we performed Western Blot analysis on a panel of MM cell lines. RB1 protein was detected in all cell lines that retained at least one *RB1* allele. U266, shown to have undergone rare biallelic loss of *RB1* [36], expressed no RB1 protein as expected, as did UTM2 cells (Figure 2). LP-1 and KMS-11 cells, which retain one copy of chromosome 13 [36], expressed lower levels of RB1 protein than OPM-2 and RPMI-8226 cells, which retain two copies of *RB1* [36]. These data suggest RB1 protein levels are related to *RB1* genomic copy number.

Given the lack of *RB1* mutations identified in this study, we hypothesized RB1 protein would be inactivated by phosphorylation in MM. All MM lines that retained at least one copy of

RB1, expressed phosphorylated (Ser807/811) RB1 protein (Figure 2) consistent with a previous analysis [13] and [37] whose levels also appeared to correlate with *RB1* copy number. These data show retained *RB1* alleles are expressed and raise the possibility that *RB1* haploinsufficiency contributes to MM/MGUS pathogenesis.

NBEA expression in MM with del[13]

We sought to validate *NBEA* as a deletion target by characterizing its expression in MM cells. We anticipated that patient samples with del[13] would have lower *NBEA* expression than patient samples without del[13]. We analyzed two large microarray data sets (Mayo GSE 6477) [26] and MMRC (<http://www.themmr.org>; total n=262) for expression changes based on chromosome 13 status. In both datasets, *NBEA* transcript levels were significantly decreased in patient samples with del[13] (Figure 4, S2; Table 3, S6, S7).

We developed a quantitative real-time reverse transcriptase PCR (Q-RT-PCR) assay to validate *NBEA* transcript expression, and assayed a panel of MM cell lines (MMCL). Some MMCL expressed low levels of *NBEA* transcript, as anticipated, but surprisingly, several MMCL expressed *NBEA* at high levels (Figure 3C). We found UTM2 cells expressed *NBEA* at levels three times higher than in a human brain sample, where *NBEA* is normally most highly expressed [38], [39], and [40]. OPM2 cells had levels 30% of brain while U266 had levels 18% of brain. RPMI-8226 and LP1 had low/undetectable transcripts (Figure 3C). We examined *NBEA* protein levels in these MMCL by Western Blotting of whole cell lysates. Consistent with the Q-RT-PCR data, we found *NBEA* protein expression varied significantly between cell lines (Figure 3D). The UTM2, OPM2, and H929 cell lines had the highest *NBEA* protein levels, while RPMI 8226, U266 and LP1 had low to undetectable *NBEA* protein.

Finally, we measured *NBEA* transcripts and protein levels in a set of CD138-enriched primary MM bone marrow samples (n=14) using Q RT PCR and Western blotting. We found *NBEA* transcript expression varied significantly across samples and, consistent with our MMCL data, some MM patients, even with del[13], harbored high *NBEA* transcript levels (Figure 3E). Because of the large number of CD138 cells needed for Western analysis we were forced to analyze a separate cohort of MM patient samples by Western blot (only sample 14216 had both RNA and protein data, and expression was low by both analyses). Consistent with the RNA data, Western blotting using *NBEA*-specific antibodies demonstrated that *NBEA* protein was strikingly dysregulated in patient MM cells (Figure 3F).

DISCUSSION

We used a novel ultra high-resolution aCGH strategy to map somatic chromosome 13 deletions with unprecedented resolution in 20 patients with MM, MGUS or amyloidosis. We used a custom CGH array dedicated solely to chromosome 13 (60bp median probe spacing) and genomic DNA from patient-matched germline (skin) biopsy samples as controls to eliminate signal noise due to DNA copy number polymorphisms [15] and [16]. We avoided noise introduced by whole genome amplification strategies by using non-amplified genomic DNA from cases with high yields of CD138+ bone marrow mononuclear cells. Patients with low bone marrow tumor burden may therefore have been underrepresented in this study. However, analysis by standard techniques of FISH and cytogenetics detected chromosome 13 deletions at expected frequencies [2] and [4] (Table 1) suggesting our patients were generally representative of other MM, MGUS and amyloidosis cohorts. Although our detection of del [13] by I-FISH was lower than other reports, our analysis was performed on non-enriched paraffin embedded bone marrow samples.

We found two regions of recurrent DNA copy number loss that were non-overlapping and mapped to two genes: *RB1*, the canonical tumor suppressor at 13q14.2, and *NBEA* at 13q13, a

gene whose role in cancer is less clear. Two independent, unsupervised analyses (CBS and Process Control) generated gene lists affected in our patient set that largely overlapped (Table 2 and S2) demonstrating the high quality of our aCGH data. Both lists included the *RB1* and *NBEA* genes. Visual inspection of log₂ plots at these loci in high-resolution and PCR confirmed the identification of *bona fide* deletion events (Figure 2–3 and data not shown). Extremely small deletions (3.49Kb and 106Kb) in a single patient (95295) significantly narrowed the MDRs we identified. In sample 95295, *NBEA* and *RB1* were the only two genes on chromosome 13 affected by DNA copy number loss. Re-sequencing analysis of the *RB1* gene within this patient sample revealed only homozygous SNPs (Table S4) demonstrating isodisomy/gene conversion across all or part of chromosome 13. These data strongly suggest that chromosome 13 DNA copy number decreases in this patient (i.e. *RB1* and/or *NBEA* loci) were selected for during disease development and likely contribute to MM biology.

Homozygous deletions of *RB1* are rare in MM [32], and we considered the possibility that 95295 might be an outlier. This patient harbored the t(4;14) translocation, and had rapidly progressive disease characterized by treatment resistance (not shown). If the 95295 sample is removed from our analysis, however, our conclusions remain substantially unchanged. Two distinct MDRs are still defined by the remaining interstitial deletions and identify a small number of candidate genes. At 13q13, *NBEA* remains the sole gene affected. Without 95295, the MDR at the gene-rich 13q14.2 locus expands to include: *SUCLA2*, *NUDT15*, *MED4*, *ITM2B*, *RB1*, *P2RY5* and *RCBTB2*. Unlike in retinoblastoma, the retained *RB1* allele is not affected by mutations in MM, so we cannot formally exclude the possibility that additional 13q14.2 genes contribute to myeloma biology.

Our expression analysis identified fourteen chromosome 13 genes with decreased expression in patient samples harboring monosomy 13, including *RB1*. Detection of decreased *RB1* levels in del[13] samples has been inconsistent in prior studies [12], [13], [14] and [42]. We found decreased levels in del[13] samples consistent with prior reports [12] and [42]. The inconsistencies are probably due to variability of *RB1* expression, which we have also observed (data not shown) or differences in criteria used in gene expression analyses. We also identified Ring finger 6 (*RNF6*) previously shown to have tumor suppressor function [43] and Integral membrane protein 2B (*ITM2B*) identified to undergo single copy number loss and promoter methylation in bladder cancer [44], both identified in a prior report to be decreased in del[13] [12]. Although a gene of interest due to prior reports of MM involvement, we did not identify *CUL4A* in the aCGH or expression analysis [12] and [13]. This does not exclude a potential role for *CUL4A* in MM biology, as MM is a disease with diverse clinical presentations and complex genetics. Further analysis is required to determine the role of these genes, if any, to myeloma biology.

Our genetic data suggest that *NBEA* may be a novel plasma cell dyscrasia tumor suppressor gene. *NBEA* shares sequence homology to the *Drosophila melanogaster* protein kinase A anchoring protein *rugose* and the mammalian gene *LRBA*. *NBEA* encodes a PKA binding domain and is a predicted PKA anchoring protein (AKAP) [39] and [40]. The C-terminal region of *NBEA* encodes a BEACH domain that is situated next to a WD40 domain, suggesting a role in protein-protein interactions. Crystal structure analysis revealed a structurally conserved PH domain intimately associated with the BEACH domain of *NBEA* [45]. *NBEA* is a large gene (730Kb) encompassing the FRA13A fragile site [46]. The breakpoints we observed were centromeric to the most fragile FRA13A breakpoint region in *NBEA*, suggesting that the *NBEA* deletion events we observed were not “bystander mutations.”

A functional role for *NBEA* was suggested by the dysregulation of its expression in MM patient samples. DNA microarray data from a large number of patient samples (n=262) demonstrated that compared to patients with normal chromosome 13, there was a decrease in *NBEA*

expression in del[13]patients. These data suggest that NBEA may be a novel tumor suppressor gene in MM. On the other hand, our Q-RT-PCR and Western blot analysis performed on a separate cohort of MM samples revealed that some patients, even with del[13], harbored very high NBEA expression (Figure 3). A prior study showed increased NBEA expression with advanced disease stage in primary plasma cell dyscrasias [47]. Furthermore, *NBEA* was one of a small set of genes whose expression was *increased* in del[13] patients in another study (supplemental data of [14]). Inactivating mutations in the p53 tumor suppressor gene is often associated with high p53 expression [48] so these data may all be consistent with a role for *NBEA* as a tumor suppressor. Sequencing of *NBEA* genes in MM patient samples will be required to confirm this hypothesis.

NBEA shares 62% sequence identity at the amino acid level to *LRBA*, a homolog also implicated in cancer cell growth [49]. Intriguingly, knock-down of *LRBA* in cancer cell lines *decreased* the growth of cells in culture, and these authors proposed that *LRBA* functions as an oncogene by facilitating EGFR [49]. Additional studies are required to elucidate the contribution of *NBEA* to MM disease biology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Array CGH identifies DNA copy number loss on chromosome 13 not detected by FISH or cytogenetics

A. Whole chromosome 13 log₂ plots of four patients with visually detectable chromosome 13 copy number loss (58762, 64511, 95295 and 68319). **B.** Pie chart summary of chromosome 13 abnormalities detected by cytogenetics, M-FISH, I FISH and/or aCGH. Eight patient samples harbored a chromosome 13 abnormality detected by aCGH. Five of these appeared normal by cytogenetic and FISH analysis. The number of patient samples is indicated in parentheses. **C.** Visual analysis of patients with interstitial deletions revealed by CBS analysis (black lines) within 23 to 50Mb (13q12-13q14.3) on chromosome 13. Figure is to scale except for deletions smaller than 150Kb, which required scaling up to be visualized. Exact sizes of all segments shown are in Table 2. Cytogenetic data was used for whole chromosome 13-deletion information (gray lines). Eight patient samples had coordinate copy number loss involving *RB1* and *NBEA* (five patient samples with whole chromosome 13 deletion and three with interstitial deletions) highlighted by vertical rectangles. Patient sample 95295 harbored interstitial deletions affecting only *RB1* and *NBEA* and defined the minimally deleted region across these eight samples.

Figure 2. High-resolution aCGH and PCR analysis confirms *RB1* is a target of recurrent interstitial deletions at 13q14.2

A. Magnified view of DNA copy number losses located at 13q14.2. The smallest region of overlap across all three patients was defined by patient 95295, and mapped to exon 20 of *RB1* (arrowhead). Locations of two genes in the region are shown for reference at the bottom. Each dot represents the average signal of ten consecutive probes. Figure includes 1244 data points spanning 1.29Mb. The region telomeric to *RB1* within sample 95295 was not called by the 600bp CBS analysis, and therefore genes within that region are not listed in (Table 2; methods). That region was called by the analysis using different window sizes (1200bp or 300bp). Genes affected were *FNDC3A*, *MLNR* and *CDAC1*. Both *RB1* and *NBEA* were called by all analyses independent of window size. **B.** PCR analysis confirms *RB1* deletion within tumor sample of patient 95295. Germline skin DNA from patient sample 95295 (Skin: S), and an independent control sample (pooled DNA isolated from blood of four normal donors, Control: C), produced the expected full-length (4.4Kb) PCR product. In contrast, CD138 purified tumor plasma cells from patient sample 95295 (Tumor: T) revealed a smaller PCR product (1.5Kb). Sequence analysis revealed the micro-deletion spanned 3486bp, which removed 2813bp of the 3' end of intron 19, all of exon 20 (146bp), and 527bp of the beginning of intron 20. In the middle of the sequencing product was a 435bp insertion with sequence identity to a region located 35Kb downstream of *RB1* on chromosome 13 that did not map to any known gene, and was situated in the opposite orientation. The full-length 4.4Kb band was not detected in the tumor sample, suggesting a homozygous deletion. Water control is shown. Size in Kb is shown on left of gel image. **C.** Real Time PCR analysis confirms *RB1* copy

number changes identified by aCGH. Control is patient 54092, with no DNA copy number changes detected by aCGH, FISH, or cytogenetics (Table 1 and S1, data not shown). Error bars are SD of three experiments each performed in triplicate. **D.** Western blot analysis of MM cell lines OPM2, RPMI 8226 (8226), KMS11, LP-1, H929, UTM2 and U266, using an antibody that detects RB1 independent of phosphorylation status (top, IF-8 antibody). HCT is colon cancer line used for a positive control. Top band is RB1. Non-specific bands are marked by an asterisk. Duplicate blots were probed with a phospho specific RB1 (Serine 807/811: P807/811) antibody (bottom). Actin was used as a loading control.

Figure 3. *NBEA* is a target of recurrent interstitial deletions at 13q13

A. Magnified view of overlapping region of DNA copy number loss across patient samples 58762, 64511 and 95295. The only gene identified within this region is *NBEA*. The region of DNA copy number decrease within patient 64511 spanned 885Kb mapping to exon 1–9 of *NBEA*. Within patient 95295, the region spanned 107Kb mapping to *NBEA* exons 3–19. Each dot represents the average signal of 10 consecutive probes as in Figure 2. Plots include 950 data points spanning a region of 0.998Mb. In patient samples 58762 and 64511 there appears to be a small region with DNA copy increase. Examination of the raw data within this region from the reference sample (skin) from all 20 patients revealed the average of these probes was below 10,000, suggesting this is not an array artifact and was also flagged by CBS algorithm.

B. Real time PCR confirms DNA copy number loss within *NBEA*. Control is patient 54092, with no DNA copy number changes detected by aCGH, FISH, or cytogenetics (Table 1 and S1, data not shown). Error bars are SD of two experiments performed in triplicate.

C. Quantitative Q-RT-PCR analysis of *NBEA* on a panel of MM cell lines. *NBEA* levels were normalized to *GAPDH* and plotted as a percentage of human brain where *NBEA* expression is known to be high.

D. Western Blot analysis of *NBEA* on a panel of human MM cell lines. Murine brain (mBrain) was used as positive control and HSP90 β is shown as loading control.

E. Quantitative RT-PCR analysis of *NBEA* on a panel CD138 purified primary patient samples including nine of the 20 patient samples included in aCGH analysis plus an additional five. White bars indicate patient samples with normal chromosome 13 status. Gray bars indicate patient samples with full or partial chromosome 13 deletions (determined by cytogenetics, FISH or aCGH analysis). *NBEA* levels were normalized to *GAPDH* and plotted as a percentage of human brain as in C.

F. Western Blot analysis on CD138 purified lysates from five primary patient samples. Due to limited sample quantity, these patients are different from the twenty included in aCGH analysis. HSP90 β was used as loading control. KMS11 is MM cell line shown since repeat analysis showed *NBEA* protein levels were low.

Table 1

Patient Characteristics and chromosome 13 status

UPN	Age	Sex	Disease	M-Ab	Deletion of Chromosome 13		
					Cyto	M-FISH	I-FISH
95295	65	M	MM (IIIa)	IgA κ light	-	-	-
92896	51	F	MM (IIIa)	IgA κ	+	ND	+
68319	75	F	MM (IIIa)	IgA κ	-	ND	-
64511	55	F	MM (IIIa)	IgA κ	-	+	-
66704	50	M	MM (IIa)	IgA κ	ND	ND	-
33172	63	M	MM (IIIa)	IgA κ	+	ND	+
54092	71	M	MM (IIIa)	Free κ Light	-	-	-
492710	58	M	MM	ND	-	-	-
802718	63	F	MM	IgA	-	-	ND
58762	74	M	MGUS	ND	-	-	-
22848	66	F	MM (Ia)	IgG κ	+	+	-
73586	67	F	MM (IIIb)	IgA κ	+	ND	-
86267	48	F	MM (IIa)	IgG κ	-	-	ND
90866	53	M	MM (Ia)	ND	-	ND	-
247748	58	M	MM	IgG κ	-	-	-
45980	56	M	MM (IIa)	IgA κ	-	ND	-
18467	70	M	MM (IIIa)	IgG κ	-	-	-
98461	61	M	MM (IIa)	IgG κ	+	-	-
10901	46	M	Amyloidosis	IgG λ	-	-	-
19367	61	F	MGUS	IgG κ	-	ND	-

UPN refers to Unique Patient Number; M-Ab: Monoclonal Antibody; Cyto: Cytogenetics; M-FISH: Metaphase FISH; I-FISH: Interphase FISH; κ: kappa; λ: Lambda; -: no abnormality found; +: abnormality found; ND: No Data

Table 2

Chromosome 13 regions with DMA copy number decrease

UPN	Start	End	Data points	Size (Mb)	Log ₂ ratio	Location	Genes
95295	34514100	34620300	77	0.106	-0.6743	13q13	<i>NBEA</i>
	47930100	47931300	3	0.001	-1.4512	13q14.2	<i>RBI</i>
64511	33657900	34546500	947	0.889	-0.2766	13q13	<i>NBEA</i>
	44793300	44824500	39	0.031	-0.3671	13q12-14	<i>TPT1</i>
	46244100	46247700	5	0.004	-0.4766	13q14.1-14.2	<i>ESD</i>
	46454100	47760300	1218	1.306	-0.4553	13q14.2	<i>SUCLA2, NUDT15, MED4, ITM2B</i>
	47760900	48469500	698	0.709	-0.7028	13q14.2	<i>RBI, P2RY5, RCBTB2, CYSCTR2, FNDC3A</i>
	48470700	48719100	204	0.248	-0.3458	13q14.2	<i>FNDC3A, MLNR</i>
	48719700	48892500	186	0.173	-0.6435	13q14.2	<i>CDADC1, CAB39L</i>
	49647300	49967100	369	0.32	-0.4726	13q14.3	
	49968900	51534300	1800	1.565	-0.439	13q14.2-14.3	<i>DLEU7, INT56, WDFY2, ATP7B, UTP14C</i>
56762	18242100	18255300	7	0.013	-0.5696	13q11	
	18401700	18412500	15	0.011	-1.017	13q11-12.11	
	18413100	18437700	34	0.025	-0.4102	13q12.11	
	18438300	18495900	62	0.058		13q12.11	
	18497700	34566900	17376	16.07	-0.3127	13q12.11-13	<i>TUBA3C, TPTE2, MPHOSPHO5, PSPC1, ZMYM5, ZMYM2, GJA3, GJB2, GJB6, CRYL1, FFT86, IL170, XPO4, LAT52, SAPI8, C10ORF3, MRP63, FGF9, SGCG, SACS, TNFRSF19, MIPF, SPATA13, PARP1, ATP12A, RNFI7, CENPJ, PABPC3, MTMR6, NUPL1, ATP8A2, RNF6, CDK8, WASF3, GPR12, USP12, RPL21, RASL11A, GTF3A, MTIF3, LNX2, DOLRID, GSX1, PDX1, CDX2, FLT3, PAN3, FLT1, POMP, SLC7A1, UBL3, KATNAL1, HMGB1, VSPL1, ALOX5AP, HSPH1, B3GALT1, RXFP2, BRCA2, PFAAP5, PD550, KL, STARD13, RFC3, NBEA</i>
	34605900	35860500	1378	1.255	-0.2939	13q13-13.3	<i>NBEA, MAB2L1, DCLK1, SPG20</i>
	46973700	48119100	1051	1.145	-0.4193	13q14.2-14.3	<i>SUCLA2, NUDT15, MED4, ITM2B, RBI, P2RY5, RCBTB2</i>
	106593300	1.07E+08	554	0.455	-0.3385	13q33.3	
	107112900	1.07E+08	43	0.032	-0.283	13q33.3	
68319	23969100	24009300	43	0.04	-0.4901	13q12.12	
	24009900	24039900	30	0.03	-0.3091	13q12.12	<i>PARP4</i>
	24250500	24406500	130	0.156	-0.4715	13q12.12	<i>RNF17, CENPJ</i>
	24492900	24767100	316	0.274	0.4239	13q12-13	<i>PABPC3, MTMR6</i>
	25064100	25500900	444	0.437	-0.5472	13q12.3	<i>POMP</i>

UPN	Start	End	Data points	Size (Mb)	Log ₂ ratio	Location	Genes
	43793100	43950900	198	0.158	-0.5347	13q14.11	<i>CI3ORF21</i>
22649	89660700	89662500	3	0.002	-1.07	13q31.3	
92896	29706900	29710500	4	0.004	-0.7369	13q12.3	<i>KATNAL1</i>
	951330900	95152500	17	0.022	-0.4041	13q32	<i>DNAJC3</i>
90665	15138300	15139500	3	0.001	-0.5112	13q11	
	15210300	15216100	14	0.005	-0.3964	13q11	
	19552700	19674700	17	0.012	-0.4905	13q12.11	
	40136700	40138500	4	0.002	-0.4942	13q14.11	<i>FOXO1</i>
	111978300	112020900	31	0.043	-0.6372	13q34	
	112194900	112196700	4	0.002	-0.6467	13q34	<i>TUBGCP3</i>
	112727100	112726300	3	0.001	-1.2328	13q34	
	112728800	112806100	117	0.079	-0.2555	13q34	<i>MCZL</i>
	112808700	112811700	5	0.003	-1.0976	13q34	<i>F7</i>
	113048700	113050500	4	0.002	-0.9167	13q34	<i>GRTP1</i>
	113126900	113120300	5	0.002	-1.4808	13q34	<i>ADPRNL1</i>
	113295300	113206500	3	0.001	-1.3918	13q34	<i>TFDPI</i>
	113498300	113407500	3	0.001	-1.3736	13q34	
88267	113327100	113328300	3	0.001	-0.7278	13q34	<i>TFDPI</i>

Patients listed displaced significant regions of copy number decreased using Circular Binary Segment Analysis (methods)

Genomic locations are based on Build 35. Genes in bold type were also denoted by Process Control Analysis (Table S2)

UPN unique patient number

Table 3

Genes with decreased expression in deletion 13 samples

Gene	Gene ID	Score(d)	Fold Change	q-value(%)
TUBGCP3	203690_at	-4.83	0.57	0
RNF6	203403_s_at	-5.68	0.60	0
GTF3A	215091_s_at	-5.77	0.62	0
UBL3	201534_s_at	-4.49	0.63	0
NBEA	221207_s_at	-3.44	0.67	0
ITM2B	217731_s_at	-4.24	0.70	0
RB1	211540_s_at	-2.92	0.74	1.3
HMGB1	216508_x_at	-4.52	0.74	0
MRP63	204387_x_at	-4.48	0.77	0
PSPC1	222612_at	-3.89	0.79	0
CRYL1	220753_s_at	-2.50	0.80	4.06
TPT1	214327_x_at	-3.84	0.85	0
ESD	228162_at	-2.83	0.89	1.72
MTMR6	228789_at	-2.63	0.90	2.73

Genes in bold were identified by Process Control and CBS analysis