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A compendium of myeloma-associated chromosomal copy number abnormalities and their prognostic value

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To obtain a comprehensive genomic profile of presenting multiple myeloma cases we performed high-resolution single nucleotide polymorphism mapping array analysis in 114 samples alongside 258 samples analyzed by U133 Plus 2.0 expression array (Affymetrix). We examined DNA copy number alterations and loss of heterozygosity (LOH) to define the spectrum of minimally deleted regions in which relevant genes of interest can be found. The most frequent deletions are located at 1p (30%), 6q (33%), 8p (25%), 12p (15%), 13q (59%), 14q (39%), 16q (35%), 17p (7%), 20 (12%), and 22 (18%). In addition, copy number-neutral LOH, or uniparental disomy, was also prevalent on 1q (8%), 16q (9%), and X (20%), and was associated with regions of gain and loss. Based on fluorescence in situ hybridization and expression quartile analysis, genes of prognostic importance were found to be located at 1p (*FAF1*, *CDKN2C*), 1q (*ANP32E*), and 17p (*TP53*). In addition, we identified common homozygously deleted genes that have functions relevant to myeloma biology. Taken together, these analyses indicate that the crucial pathways in myeloma pathogenesis include the nuclear factor-κB pathway, apoptosis, cell-cycle regulation, Wnt signaling, and histone modifications. This study was registered at http://isrctn.org as ISRCTN68454111. (*Blood*. 2010;116(15): e56-e65)

Introduction

Myeloma is thought to result from the transformation of a proliferative "plasmablastic" cell located in the germinal center.[1] The progeny of this cell migrate to specialized niches in the bone marrow where they mature toward terminally differentiated antibody secreting plasma cells. Genetic alterations in the myeloma clone can impact on pathways important in normal plasma cell biology leading to significant changes in cellular behavior, disease progression and changes in clinical outcome. Cytogenetics and positional cloning have given many insights into myeloma pathogenesis and the characterization of abnormalities focused attention on recurrent chromosomal translocations and cyclin D deregulation as a classification system.[2]

Additional information about the genetics underlying the deregulation of normal plasma cell functional pathways is being derived from the characterization of the regions of recurrent copy number alteration (CNA), which are frequent in myeloma compared with other hematologic malignancies, and we have previously reported on the importance of deletions of *CDKN2C* at 1p32 impacting on the G_1/S transition.[3,4] High-resolution single nucleotide polymorphism (SNP)–based gene mapping is a tool that is highly effective in this respect and when combined with expression and mutation analysis can identify candidate genes important in myeloma pathogenesis, as has been shown for other tumor types.[5] Using this approach we identified the importance of *CYLD* deletion and mutation while others identified deletion of *TRAF2*, *BIRC2*, and *BIRC3* that when considered together are consistent with activation of the nuclear factor-κB (NF-κB) pathway in a proportion of myeloma cases.[6-8]

Information on CNAs in myeloma samples have been reported using karyotyping, fluorescence in situ hybridization (FISH), array comparative genomic hybridization (aCGH), and SNP-based arrays.[6,8-13] However, to date there have been no detailed reports of CNAs in myeloma. Here we describe and annotate the major CNAs found in a large series of myeloma samples. We have analyzed gains and deletions in presenting myeloma samples using high resolution SNP-based mapping arrays with which expression array data have been integrated to delineate genes whose expression may be affected by these abnormalities. We have extended this work further by examining the clinical importance of relevant lesions in a large set of uniformly treated clinical samples from the Medical Research Council (MRC) Myeloma IX study.

Methods

Patient samples

Bone marrow aspirates were obtained from newly diagnosed patients with multiple myeloma, entered into the UK MRC Myeloma IX study, after informed consent. Plasma cells (PCs) were selected to a purity of > 90% using CD138 microbeads and magnet-assisted cell sorting (Miltenyi Biotec) and analyzed by FISH (n = 1177), expression array (n = 258), and/or mapping array (n = 114).[14] Sample characteristics are summarized

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Table 1. Patient characteristics for mapping, expression, and total datasets

Characteristic	Mapping (n = 114)	Expression (n = 258)	Myeloma IX (n = 1966)
Age, y			
Mean	64.3	64.7	64.6
SD	10.0	10.0	10.2
Serum β2m, mg/L			
Mean	6.0	5.9	6.1
SD	4.0	4.3	5.9
Total no. patients tested	80	190	1789
Platelets, 10^9/L			
Mean	253.3	247.0	247.5
SD	99.0	92.0	97.3
Total no. patients tested	114	258	1880
Hemoglobin, g/dL			
Mean	10.2	10.5	10.8
SD	1.9	1.9	4.3
Total no. patients tested	114	258	1880
Serum albumin, g/L			
Mean	34.8	34.2	34.7
SD	7.2	7.3	7.0
Total no. patients tested	114	257	1858
Deletion 13q			
Percent	52.5	45.4	45.3
No. total patients	53	109	473
Total no. patients tested	101	240	1043
t(4;14)			
Percent	16.7	15.8	11.4
No. total patients	17	38	120
Total no. patients tested	102	240	1052
t(11;14)			
Percent	17.5	17.8	13.9
No. total patients	18	43	146
Total no. patients tested	103	241	1047
ISS, % of patients			
1	20.8	22.3	22.0
2	35.0	38.5	39.4
3	44.2	39.1	38.7

ISS indicates International Staging System.

in Table 1. RNA and DNA were extracted using commercially available kits (RNA/DNA mini kit or Allprep kit; QIAGEN) according to manufacturers' instructions. Matched germline DNA from 80 patients was extracted from peripheral white blood cells, using the Flexigene kit (QIAGEN).

FISH analysis

Interphase FISH analysis was performed on CD138-selected PCs using the micro-FISH technique and probes that have previously been documented.[15-17] Briefly, probes to detect t(4;14), t(6;14), t(11;14), t(14;16), t(14;20), del(1p32.3), gain 1q, del(17p), and hyperdiploidy (essentially defined by gain of any 2 of chromosomes 5, 9, and 15) were used to identify abnormalities. In addition, analysis of 8p was performed using the Provision probes (Abbott Molecular) that interrogate 8p22 (*LPL*), 8p11.1-q11.1 (centromere), and 8q24.2-q24.3 (*MYC*).

Array analysis

GeneChip Mapping 500K Array set and U133 Plus 2.0 expression arrays (Affymetrix) were performed as previously described.[3,4,7,18] Nine samples in this study had also been analyzed in a previous 50K mapping array dataset.[3] Expression array raw data were normalized in GeneSpring 7.3 (Agilent Technologies) using per chip normalization to the 50th percentile and per gene normalization to the median. Comparisons between samples with a CNA and those without were performed using a 1-way analysis of variance and filtered to remove those with fold change < 1.2. Gene lists were further filtered to solely include those within the region of CNA, thus identifying genes whose expression is directly affected by the alteration. For mapping array data the SNP genotypes and inferred copy number were obtained using GTYPE and dChip, exactly as previously described.[4,7] Data have been deposited into GEO under accession number GSE21349. Homozygous deletions were identified as having an inferred copy number < 0.7, hemizygous deletions between 0.7-1.6 and gains > 2.4.

Clinical data

The MRC Myeloma IX trial recruited 1970 newly diagnosed patients and comprised 2 patient pathways; the first for older and less fit patients and the second for younger, fitter patients. Younger, fitter patients received autologous transplantation after induction with cyclophosphamide, thalidomide, and dexamethasone (CTD) or cyclophosphamide, vincristine, doxorubicin, and dexamethasone (CVAD), deemed the intensive pathway. The nonintensive pathway consisted of a randomization to either attenuated CTD (CTDa) or melphalan and prednisolone (MP). All patients were then randomized to thalidomide maintenance or no thalidomide maintenance. The trial was approved by the MRC Leukaemia Data Monitoring and Ethics committee (MREC 02/8/95, ISRCTN68454111).

Kaplan-Meier overall survival (OS) curves were calculated using Bioconductor and the survival package[19] after a median follow-up of 3.7 years. The difference between curves was tested using the log-rank test within Bioconductor. A threshold of significance was taken as $P < .05$.

Results

Presenting myeloma cases were studied using the 500K SNP-based mapping GeneChip (n = 114), of which 80 had matched nontumor DNA available to compute accurate CNAs and acquired loss of heterozygosity (LOH) in the tumor (Figure 1 and Table 2). We went on to determine whether these common CNAs cosegregate as well as their impact on prognosis (Table 3). Detailed analysis of the regions of loss and gain can be found in supplemental Figure 1 (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

In agreement with previous observations[20] there was an association between IgH translocations and del(13q) (χ^2 $P = 5.33 \times 10^{-24}$), an association of 1q gain with del(13q) (χ^2 $P = .000429$), an inverse association of 1q gain with gain of 11 (χ^2 $P = .000575$), and an inverse association of del(13q) with gain of 11 (χ^2 $P = .000184$). There was an association of t(4;14) with del(18) (χ^2 $P = .001865$) and an inverse association of t(4;14) with del(16q) (χ^2 $P = .00\,287$).

Gain of the odd-numbered chromosomes is a characteristic of hyperdiploid myeloma and when ranked by the number of full gains chromosome 15 was most often gained (42/53 samples), followed by chromosomes 9 (41 samples), 5 (38 samples), 19 (38 samples), 3 (31 samples), 11 (28 samples), 7 (24 samples), and 21 (14 samples). In a larger dataset of 1004 samples, hyperdiploidy, as determined by FISH, was not associated with a significantly better OS when compared against all nonhyperdiploid samples (49.7 vs 43.7 months, $P = .15$, supplemental Figure 2). In addition, gain of chromosome 5 did not stratify hyperdiploid cases, because cases with and without the abnormality showed similar OS times (William J. Tapper, L.C., G.P.D., Z.J.K., D.M.S., Alex J. Szubert, W.M.G., Sue E. Bell, Graham H. Jackson, J. Anthony Child, G.J.M., F.M.R., manuscript in preparation). We were able to divide hyperdiploid samples into those with gain of chromosome 11 and those with gain 1q and del(13q). A clear clinical disadvantage was seen in hyperdiploid samples with gain 1q, with or without del(13q), compared with those with normal 1q (supplemental Figure 2), as has been previously reported.[20]

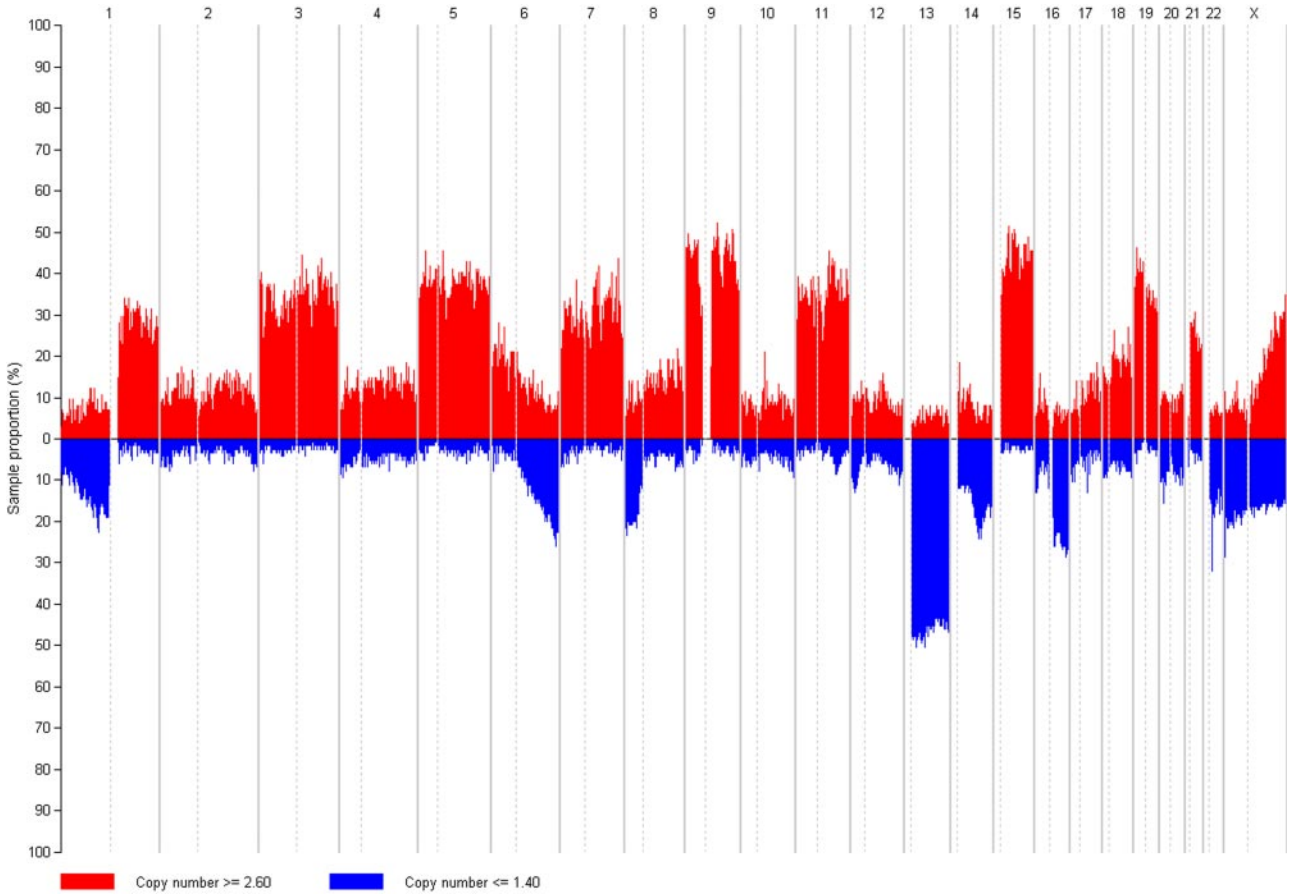

Figure 1. Summary plot of gains and deletions in 114 myeloma samples. Output from dChip (August 2008 Version) showing chromosomes along the x-axis and frequency of abnormality along the y-axis. Gains in red and deletions in blue.

CNAs with prognostic significance

1p. In this dataset 34 cases (29.8%) had any deletion of 1p, of which 29 had an interstitial deletion, and 5 had the whole arm deleted. Copy number–neutral loss of heterozygosity (LOH), or uniparental disomy (UPD), was seen on 1p with 3 cases having complete UPD of 1p and an additional 2 cases with partial UPD extending from 1p36.13 and 1p36.12 to the telomere.

There were 4 minimally altered regions on 1p. We have previously identified the region at 1p32.3, containing *FAF1* and *CDKN2C*, which is associated with adverse overall survival (OS)[4] and had a deletion in 18 samples. In line with these observations, 6 cases in this dataset had homozygous deletion of both *CDKN2C* and *FAF1* and could not delineate which gene was the target (Figure 2A); however, others have proposed *CDKN2C* as the sole target.[21] Twelve additional cases had hemizygous deletion. Confirming our previous study of this region we expanded this analysis further using FISH to examine 510 newly presenting cases in the intensive path of the trial and show a significant effect on OS (Figure 3A, $P < .001$, median OS 34.5 vs > 70 months). It is not clear whether the effect on survival is solely due to deletion of 1p32.3 as cases may also have additional deletions affecting other regions and genes on 1p.

Table 2. Summary of deletions and gains in 114 myeloma samples

Chromosome	Deletions (%)	Chromosome	Gains (%)
1p	29.8 + 4.4 UPD	1q	36.0
6q	33.3	3	27.2
8p	25.4	5	33.3
12	21.9	7	21
13q	58.7	9	35.9
14q	38.1	11	24.6
16q	35.0 + 8.7 UPD	15	36.8
17p	7.0 + 1.75 UPD	19	33.3
18	15.8	21	12.3
20	12.3		
22	18.4		
X	28.0 + 21.0 UPD	X	8.7

UPD indicates uniparental disomy.

The most commonly deleted region on 1p was at 1p22.1-p21.3 that contains 35 genes and was deleted in 25 cases (22%). Comparison of expression data from deleted and nondeleted samples showed that 2 adjacent genes, *MTF2*, and *TMED5*, were down-regulated in this region. Another deleted region lies at 1p12, and was hemizygously deleted in 20 cases, with homozygous deletion of the *FAM46C* locus in an additional 2 cases. A fourth deleted region lies within 1p31.1 and encompasses 6.6 Mb containing 14 genes with only 1 gene underexpressed in deleted samples, *USP33*, a ubiquitin-specific protease.

1q. Gain of 1q was identified in 39/114 (34.2%) samples of which 2 had complete LOH. In myeloma, gain of 1q has an adverse effect on OS[22] and in this data series, studying 531 samples in the intensive arm of the trial using a FISH probe specific for *CKS1B*, this was confirmed ($P < .001$, median OS 52.1 vs > 70 months, Figure 3B). This effect was examined further by removing cases

Table 3. Summary of prognostic regions and genes tested

Copy number abnormality	Prognostic significance	Genes identified	Gene function
del(1p)	Yes	*FAF1**	Fas associated
		*CDKN2C**	Cell-cycle inhibitor
1q+	Yes	*CKS1B**	Cyclin-dependent kinase
		ANP32E†	Histone acetyltransferase inhibitor
del(8p)	No	NA	NA
del(13q)	No‡	NA	NA
del(16q)	No	NA	NA
del(17p)	Yes	*TP53**	Regulator of transcription

NA indicates not applicable.
*Significant by fluorescence in situ hybridization (FISH).
†Significant by expression quartile analysis.
‡Not significant by FISH when del(17p), t(4;14), t(14;16), and t(14;20) samples are removed from the analysis.

with other adverse cytogenetic factors (del(17p) and translocations involving *FGFR3*/*MMSET*, *MAF*, and *MAFB*) after which gain of 1q still retained prognostic significance ($P = .01$, median OS 57.8 vs > 70 months, supplemental Figure 2), suggesting that gain of 1q21 is an independent prognostic factor.

One case had gain of all of 1q but with acquired LOH between 1q21.1-q25.2, and an additional 5 cases had UPD of all of 1q (2 of which were UPD of the entire chromosome). A minimally amplified region was identified between 1q21.1-q23.3 containing 679 genes. When expression array data were clustered according to copy number status (2 vs > 2 copies), 9 genes had a greater than 2-fold increase in expression including *IL6R*, *CKS1B*, *KCNN3*, *ANP32E*, *FCRL2*, *S100A4*, *HAX1*, *PEX11B*, and *C1orf43*. Of these, *IL6R*, *KCNN3*, and *CKS1B* had a greater than 2.5-fold increase in expression. The expression data for these genes was ranked in 258 samples and the lowest expressing quartile compared with the highest expressing quartile and Kaplan-Meier survival curves generated for each gene. From this analysis, only *ANP32E* had a

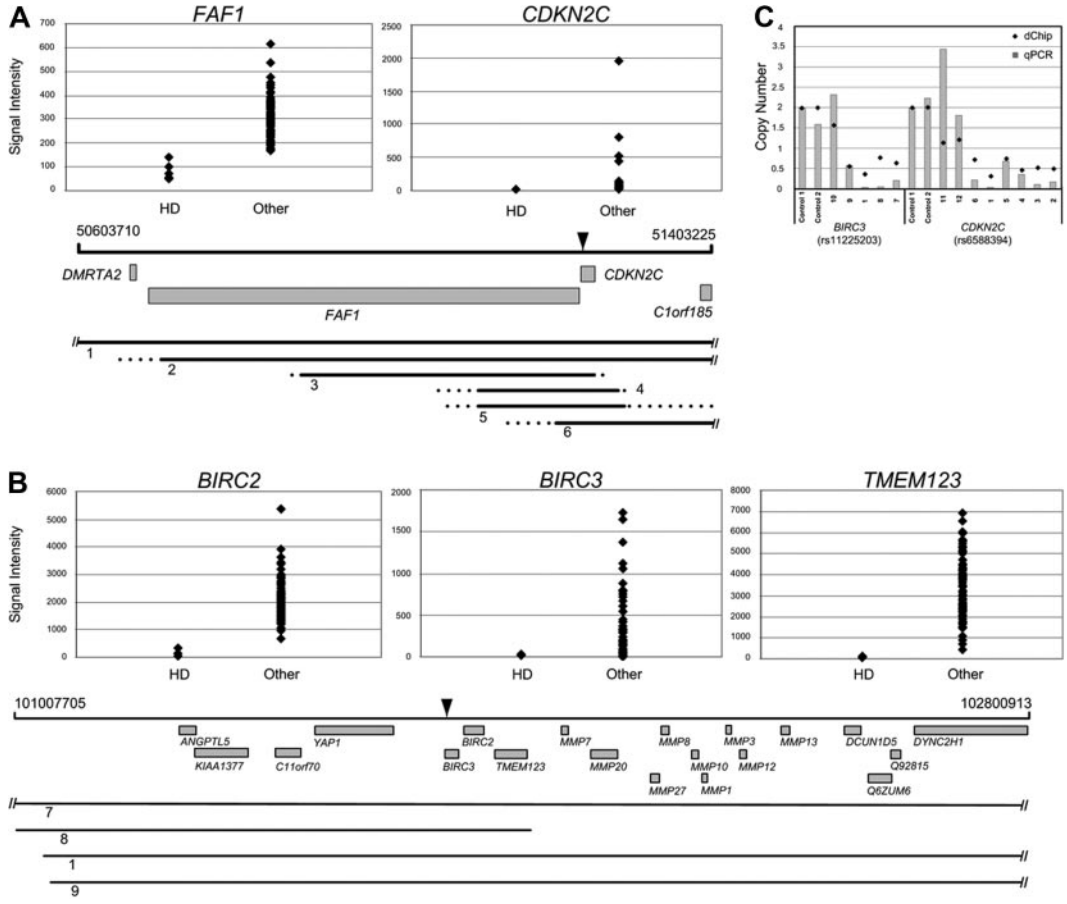

Figure 2. Homozygous deletions in *CDKN2C*/*FAF1* and *BIRC2*/*3* with expression data. (A) Expression plots for *FAF1* and *CDKN2C* showing expression levels in samples with homozygous deletion (HD) and in samples without homozygous deletion (Other). The genomic locations of *FAF1*, *CDKN2C*, and surrounding genes are shown to scale with the region deleted in each sample indicated by solid lines below. Dotted lines indicate the region between SNPs that may or may not be deleted. Forward slashes indicate that deletions extend beyond the region shown. The downward arrow indicates the position of TaqMan primers used for gene dosage validation. (B) As in panel A, but the region shows the positions of homozygous deletions on 11q surrounding *BIRC2* and *BIRC3*. (C) Gene dosage validation of homozygous deletions by TaqMan showing copy number identified by both array (dChip) and TaqMan (quantitative PCR). Sample numbers are shown along the x-axis with control samples.

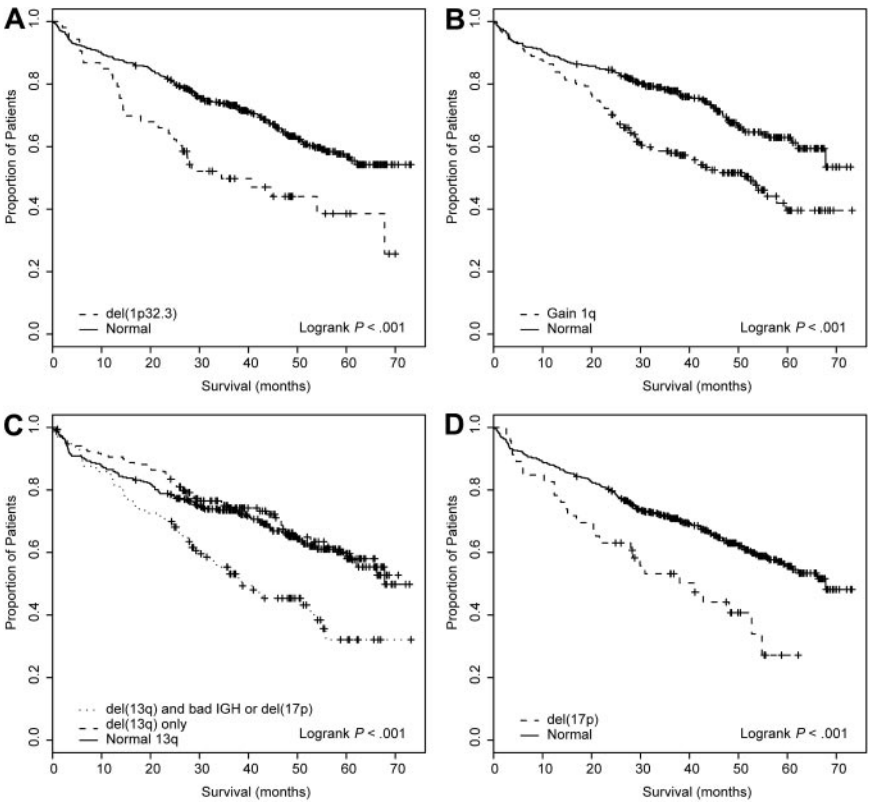

Figure 3. Effect of chromosomal abnormality on survival in Myeloma IX patients. The effect of loss of 1p32.3 (*FAF1*/*CDKN2C* n = 866, A), gain of 1q (*CKS1B* n = 909, B), deletion of 13q (*RB1* n = 1058, C), and deletion of 17p (*TP53* n = 1016, D) on overall survival (months).

statistically significant effect on OS ($P = .004$, median survival 28.9 vs 58.9 months), and validated in another dataset (GSE2658, supplemental Figure 3).[23]

Chromosome 17. Deletion of 17p was seen in 8 samples (7%) with UPD in a further 2 samples. One sample had a small deletion of 0.55 Mb containing 30 genes centered on *TP53*. Expression of *TP53* in the del(17p) samples was significantly lower than in nondeleted samples. Four further genes within the minimally deleted region (MDR) were expressed at lower levels in the deleted samples: *TMEM107* (2.2-fold), *MGC10744* (1.8-fold), *SAT2* (1.4-fold), and *EIF5A* (1.3-fold). Loss of 17p (using a *TP53* FISH probe, n = 501) was associated with an adverse OS (median OS 40.9 vs 67.8 months, $P < .001$, Figure 3D) in the intensive arm of the trial. We did not identify homozygous deletions of *TP53*, but mutation analysis of cases with LOH identified *TP53* mutations in 27% of the samples (K.D.B., F.M.R., L.C., William J. Tapper, G.P.D., R.K.M.P., D.M.S., Z.J.K., W.M.G., B.A.W., Christopher P. Wardell, D.G., F.E.D., G.J.M., manuscript in preparation), directly implicating *TP53* as the crucial gene at this site.

CNAs in chromosomes associated with recurring immunoglobulin rearrangements

Chromosome 4. The most frequently deleted region on chromosome 4 was of the telomere of the short arm as a consequence of the unbalanced t(4;14) translocation that abrogates *FGFR3* expression. Of the 17 samples with t(4;14), 4 had deletion of the tip of the short arm (4p16.3) at the *FGFR3* locus (Figure 4A-B). An *IgH-MMSET* cDNA polymerase chain reaction (PCR) showed that all but 1 sample with del(4p16.3) had an MMSET translocation product (Figure 4C). The remaining sample did not give an *IgH-MMSET* cDNA product but the mapping data indicate that the breakpoint in this sample is within *FGFR3*, consistent with a longer cDNA product. A copy number breakpoint at the *FGFR3* locus resulted in loss of expression of *FGFR3* (Figure 4D).

We found deletion of the reciprocal translocation chromosome 14 in all cases with deletion of *FGFR3*. However, the *IGH* locus to the telomere retained a normal copy number, confirming that the der(14) chromosome is lost after the translocation occurs (Figure 4E). This results in loss of the *FGFR3* allele that is under the control of the *IGH* enhancer.

Chromosome 8. Deletion of 8q was present in 14% and included 3 samples with deletion of all of chromosome 8, but most deletions were small and interstitial. There were 10 samples (8.7%) with a copy number breakpoint at the *MYC* locus, 5 of which were small regions of gain and may be due to a translocation or gene duplication. Expression of *MYC* in these samples was high compared with those without breakpoints, indicating that the CNAs directly affect expression of the oncogene.

Also on chromosome 8, between 75% and 100% of 8p was deleted in 29 of 114 (25%) samples. The use of 2 of the interstitial deletions allowed the identification of a 23.1 Mb MDR between 8p23.1-p12, containing 237 genes. By differential expression analysis these 237 genes were limited to 36 genes that are underexpressed (> 1.2-fold, $P < .05$) and of these 14 are underexpressed > 1.5-fold. The most underexpressed genes are *ZDHHC2* (1.9-fold), *FDFT1* (1.8-fold), *CNOT7* (1.8-fold), *PPP2R2A* (1.7-fold), and *PPP2CB* (1.6-fold). FISH for del(8p) was performed on 152 presenting cases, and while it was deleted in 29% of samples it did not impact on survival in this dataset (supplemental Figure 2).

Chromosome 11. Gains of chromosome 11 were common (47/114, 41%), and 31 cases had gain of the whole chromosome.

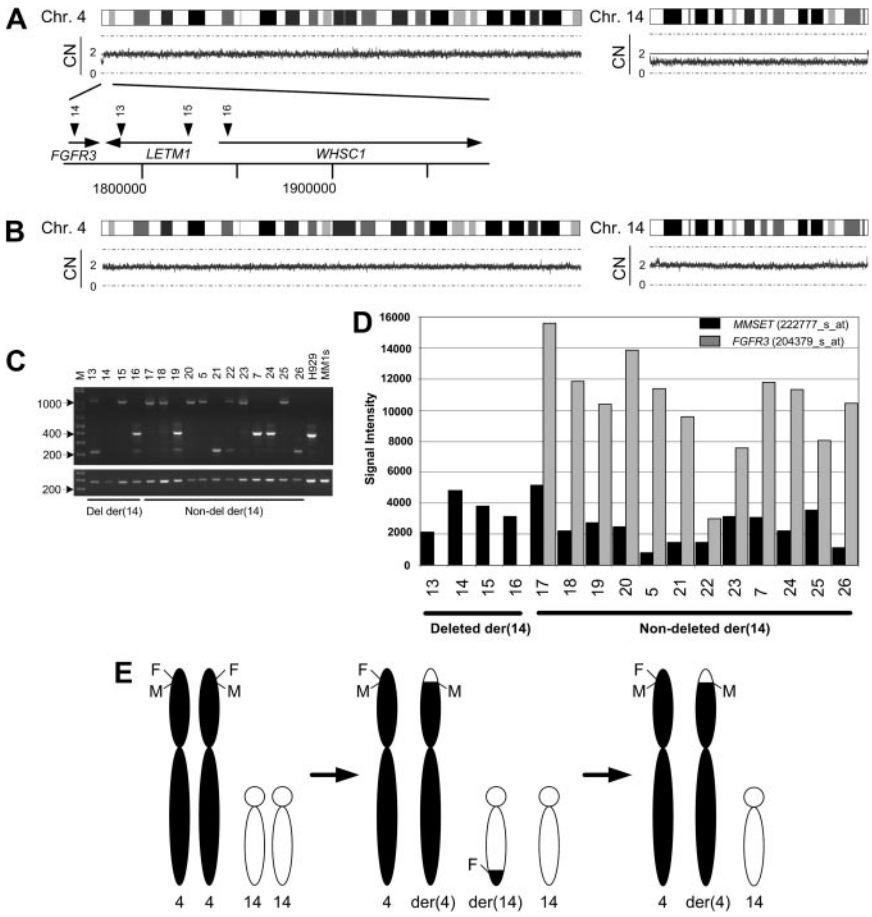

Figure 4. Loss of expression of *FGFR3* in t(4;14) myeloma is a result of loss of der(14). (A) A copy number plot output from CNAG showing an example of a t(4;14) sample without expression of *FGFR3* with decreased copy number at 4p16-ptel on chromosome 4 and on chromosome 14. The enlarged area shows the approximate positions of the breakpoints in the 4 samples without *FGFR3* expression (down arrows). (B) As above but for a t(4;14) sample with *FGFR3* over-expression. (C top panel) *IgH-MMSET* cDNA products from each t(4;14) sample, with or without loss of der(14), and controls H929 (t(4;14) positive) and MM1s (t(4;14) negative). Bands at 1000, 400 and 200 bp correspond to MB4-1, MB4-2 and MB4-3 breakpoint products. (Bottom panel) *ABL* control cDNA PCR. (D) Expression levels of *FGFR3* and *MMSET* in the t(4;14) samples by microarray. (E) A model explaining the loss of *FGFR3* expression in a sample with a t(4;14) translocation through deletion of der(14) chromosome (see "Results" for details). F = *FGFR3* locus, M = *MMSET* locus.

7 samples had copy number increases at the *CCND1* locus, of which 6 had a t(11;14) suggesting they occur during the translocation event. Loss of der(14) was not seen in these samples and suggests that CNAs in this region occur through more complex chromosomal rearrangements compared with CNAs surrounding the t(4;14) breakpoint.

Additional abnormalities on chromosome 11 center on the *BIRC2*/*BIRC3* loci, where 8 samples (7%) had deletion and of these 6 had homozygous deletions. Analysis of the 7 genes (*ANGPTL5*, *KIAA1377*, *C11orf70*, *YAP1*, *BIRC3*, *BIRC2*, *TMEM123*) within the homozygously deleted region indicates that *BIRC2*, *BIRC3*, and *TMEM123* are the likely targets as *KIAA1377*, *C11orf70* and *YAP1* were not expressed in any myeloma sample (Figure 2B). Recent data focusing on the NF-κB pathway suggest that *BIRC2* and *BIRC3* are the most likely targets of the deletion.[8]

Chromosome 14. The whole of chromosome 14 was deleted in 14 samples (12%), with an additional 18 samples having smaller deletions, and a further 15 samples with deletion of 14q32.33-qter due to loss of the *IGH* locus. An 11.86 Mb MDR existed at 14q24.1-q31.1 containing 103 genes between *WDR22* and *C14orf145*. Of these 103 genes, 33 were underexpressed in the 27 samples with deletion including *ACOT2*, *ZNF410*, *SFRS5*, *SNW1*, *PSEN1*, *WDR21A*, *C14orf156*, and *ALKBH1* that were under-expressed > 1.5-fold. Homozygous deletion of *TRAF3* and *AMN*, outside of the MDR, was seen in 2 samples and another 17 samples had hemizygous deletion but had no effect on OS.

Chromosome 16. The t(14;16) translocation results in over-expression of *MAF* and disruption of *WWOX* that is located at the fragile site FRA16D. The *WWOX* locus was deleted in 33 samples with UPD in 10 additional samples. Differential expression analysis between deleted and nondeleted samples showed that deletion of 16q had the largest effect on *WWOX* with a 2.4-fold reduction in expression. *CHD9*, *MAF*, and *CDH1* were also affected > 1.6-fold.

However, *WWOX* and *MAF* are not the sole targets of CNAs on chromosome 16. Chromosome 16 was fully deleted in 4 samples with the entire long arm deleted in a further 21 samples and interstitial deletion of 16q in another 15 samples. UPD of 16q was present in 10 samples of which 3 also had UPD of 16p. These data are consistent with alterations of 16q being present in 43% of the total cases. Several regions of interest on 16q include 16q12.1 that was deleted in 33 samples with UPD present in 8 additional samples. The smallest region of LOH at this locus was a hemizygous deletion of 3.05 Mb that contained a homozygous deletion of 442 kb containing 4 genes: *NKD1*, *SNX20*, *NOD2*, and *CYLD*, of which only *CYLD* was differentially expressed (1.6-fold). Another sample had a homozygous deletion of 212 kb containing only *NOD2* and *CYLD*. These data are consistent with involvement of *CYLD*, a tumor suppressor gene and negative regulator of the NF-κB pathway, as the critical gene within this region and is mutated in 21% of samples with LOH.[6-8]

The second region is present at 16q22.1, comprises 1.05 Mb of DNA, and was deleted in 31 samples with UPD in 10 samples. This region contained 25 genes, including E-cadherin, and of these genes 9 were differentially expressed between 1.21- to 1.5-fold lower in deleted samples: *THAP11*, *NUTF2*, *EDC4*, *PSMB10*, *DPEP2*, *NFATC3*, *SLC7A6*, *SLC7A60S*, and *ZFP90*.

Other common regions of CNAs

Chromosome 6. Deletions on chromosome 6 were observed in 40/114 cases (35%) and predominantly found on 6q (38/40, 33.3%) with only 2 samples having deletion of the entire chromosome. The most commonly deleted region centered at 6q25.3 (155.35-161.74 Mb) that was deleted in 34 samples. This region contains 32 genes between *TIAM2* and *PARK2*, the location of the fragile site FRA6E. Differential expression of these samples shows 17 genes within this region had significant differences ($P < .05$) with a fold change > 1.4. Of the 17 genes, the 3 with the greatest reduction in expression are *IGF2R* (1.9-fold), *TFB1M* (1.68-fold), and *WTAP* (1.7-fold).

Gain on chromosome 6 was seen in 26/114 cases (22.8%) and was predominantly limited to 6p (21/26) with gain of the whole chromosome in 5 samples. Gains of 6p mostly extended over the entire short arm, but a minimally amplified region of 3.1 Mb was identified, at 6p22.3, and was gained in 20 samples. This region contains 9 genes, but expression analysis of these samples showed no significant overexpression of any of the genes. The next commonly gained region extended from 6p22.3-p21.31, was 20.5 Mb and was gained in 19 samples. This larger region contains 657 genes and extends from *JARID2* through histone locus 1 to *SCUBE3* within the MHC region; 34 of these genes are up-regulated in samples with gain of this region compared with those without.

Chromosome 12. On chromosome 12, 4 samples had a gain, 2 samples had UPD, and 25 samples had a deletion. The most frequently deleted region (16/114, 14%) was at 12p13.1-p13.2 and contained 49 genes including *CDKN1B*, a negative regulator of cell cycle. However, analysis between samples with and without a deletion highlighted only 3 differentially expressed genes, *CREBL2*, *ETV6*, and *MANSC1*, with > 1.5-fold decrease in expression. In this limited dataset no prognostic value of del(12p) was seen. The second most frequently deleted region (9/114, 7.9%) lies at 12q23.2 and contained 3 genes, *IGF1*, *PAH* and *ASCL1*, within a minimally deleted region of 696 kb, but none were underexpressed in the deleted samples. Unlike a previous study[10] we found no prognostic significance of del(12p) in a cohort of 803 samples examined by FISH (William J. Tapper, L.C., G.P.D., Z.J.K., D.M.S., Alex J. Szubert, W.M.G., Sue E. Bell, Graham H. Jackson, J. Anthony Child, G.J.M., F.M.R., manuscript in preparation).

Chromosome 13. Chromosome 13 has been extensively investigated both as a prognostic factor and as a location for tumor suppressor genes. In this dataset deletions were seen in 67/114 samples (59%) of which 87% (58/67) were of the complete long arm. UPD of chromosome 13 was seen in 2 samples and only 1 sample had gain of the chromosome. An MDR defined by 4 samples with interstitial deletion lay between 13q14.11-q14.3 (8.8 Mb) and contained 68 genes. Of these, 16 were significantly underexpressed in the deleted samples, and *RB1* was the most underexpressed (1.9-fold), followed by *EBPL* (1.7-fold), *RNASEH2B* (1.6-fold), and *RCBTB2* (1.6-fold). This region also contained the microRNA genes mir-16-1 and mir-15a, which may also be critical in the pathogenesis of the disease. Homozygous deletions were seen in 4 samples, but only 1 of these fell within the MDR containing *RB1*, *P2RY5*, and *RCBTB2*, and was 213.5 kb in length. The 3 remaining homozygous deletions contained no known genes. Using FISH probes for the *RB1* and D13S319 loci in 1058 presenting samples we could not define an impact on survival for del(13q) when del(17p) and t(4;14) samples were excluded (Figure 3C).

Chromosome 20. Deletions of chromosome 20 were most often seen in the short arm (14 samples) and were deletions to the telomere. Deletions of the long arm were mostly interstitial with no discernible MDR. Loss or gain of the whole chromosome was seen in 4 samples and an additional 13 samples had varying sizes of interstitial gain.

Chromosome 22. Chromosome 22 was completely deleted in 12 cases with an additional 9 cases having interstitial deletions. The *IGL* locus was also deleted in another 15 cases indicating rearranged Ig light chains. Of those with deletions at *IGL*, 4 had a homozygous deletion. One sample had UPD of chromosome 22 and 1 sample had gain of the chromosome. Another 11 samples had interstitial gains.

Chromosome X. Changes in the X chromosome are of interest because of the obvious sex differences and the potential for imprinting. In this context UPD of X was common both in males and females and is a potentially important mechanism of gene inactivation. One copy of X was lost in 19 female samples, but 4 of these had regions of gain on the remaining copy, resulting in UPD. In contrast, 18 male samples had UPD, resulting from duplication, extending from the telomere of the long arm and 2 samples had UPD of the whole chromosome (1 of each sex). Of the females, 9 samples had gain of X that also extended from the telomere of the long arm. Deletion of the telomeric end of the short arm was seen in 6 female samples. Providing further evidence for the importance of genes carried on the X chromosome, homozygous deletions were seen affecting *UTX* (histone demethylase), *CNKSR2* (regulator of Ras signaling), and *HDHD1A* (halogenase).

Discussion

This study has analyzed the myeloma genome to a resolution of 2.5 kb and identified CNAs including gains, deletions and acquired UPD. The common CNAs were found at 1p, 1q, 6q, 8p, 11q, 13q, 14q, 16q, 17p, 20, and 22, along with gain of the odd numbered chromosomes (Figure 1).

UPD as a mechanism underlying myeloma pathogenesis

UPD is a novel pathogenic mechanism affecting gene copy number and function and was frequently found at 1q (8%), 16q (9%), and X (20%) in myeloma. There are potential candidate oncogenes or tumor suppressor genes at 1q and 16q, and the frequency of UPD on Xq suggests it too may contain relevant genes. The frequency with which UPD is seen in these regions suggests that they must be under some degree of positive selection. In contrast, UPD is rarely seen in other regions of deletion such as 8p, 6q, and 13q, suggesting 2 distinct cellular mechanisms within myeloma.

UPD at 16q is frequent and is also the site of recurrent hemizygous deletions. We hypothesize that an active tumor suppressor gene allele on 16q is deleted and the remaining mutated or inactive allele is duplicated. Consistent with this hypothesis we have identified 2 tumor suppressor genes; *CYLD* and *WWOX*[7] and found mutations in the residual *CYLD* allele, present in 21% of cases with LOH (data not shown). *WWOX* is underexpressed in samples with LOH and can also be inactivated by the t(14;16) translocation.

We found 9 samples (8%) with UPD of 1q, even though this region has gains in copy number, which is unusual in cancer cells as UPD is normally associated with regions of deletion. Extra copies of 1q are frequently involved in translocations, although in the case

of UPD it may be that isochromosomes are formed in a fashion similar to follicular lymphoma where gains, UPD and isochromosome 6p are detected.[24-26] Isochromosome 1q is known to occur in other cancer types and has been proposed as a marker for aggressive tumors.[27-29]

On the X chromosome, UPD occurs through deletion of 1 copy in females and duplication of the remaining copy and in males the sole copy is duplicated. However, translocations involving the X chromosome have been reported in myeloma and the UPD that extends from the telomere of the long arm may be a result of unbalanced translocations of the region (data not shown).

The pathologic consequences of immunoglobulin gene rearrangements

A further common and important mechanism contributing to myeloma pathogenesis are translocations involving the immunoglobulin locus. In this dataset CNAs are frequently associated with translocations including 4p, 11q, 16q, and 20q. Deletion of 4p occurs in ~ 25% of t(4;14) samples resulting in loss of the over-expressed *FGFR3* allele.[30] Deletion and loss of expression correlates exactly with loss of 1 copy of chromosome 14 (with the exception of 14q32.2-qtel) confirming that the method of loss is through deletion of the der(14) chromosome. This is further evidence that the IgH translocation is the primary event and subsequently der(14) is deleted. We have identified evidence for an impact of *IGH* translocation on chromosomal abnormalities with the *MYC* locus at 8q affected in 9% of samples, *CCND1* at 11q (6%), *CCND3* at 6p (4%), and *FGFR3*/*MMSET* at 4p (6%), identifying 25% of samples with common CNAs associated with translocations. Potentially, non-Ig translocations may affect progression of multiple myeloma, but solely using SNP mapping arrays we cannot identify these and further information on these await alternate technologies.

Regions of prognostic importance

Our analysis of the regions with recurrent CNAs reveals that not all regions are of prognostic importance. Those with a prognostic impact on OS include del(1p) (*FAF1*, *CDKN2C*), 1q+ (*ANP32E*, *CKS1B*), and del(17p) (*TP53*), as well as the translocation groups t(4;14), t(14;16) and t(14;20) (Table 3). No prognostic impact on OS was found due to del(8p), del(11q), del(12p), del(13q), or del(16q). However, the importance of these latter regions should not be discounted, as some of the datasets were relatively small and were not studied extensively by FISH. The identification of novel genes, which may be important in identifying patients with a poor prognosis, merits further screening of samples for mutational and methylation abnormalities.

In this dataset, gain of the region around *CKS1B* on 1q by FISH is associated with shorter OS. This region is large and we identified *ANP32E* as a potentially interesting oncogene, whose increased expression was associated with poor OS. *ANP32E* is a member of the acid nuclear protein family that has been implicated in histone acetyltransferase inhibitory activity with a role in chromatin remodeling and transcriptional regulation, having similar properties to the more fully characterized and pathogenically important *MMSET*.[31]

Impact of homozygous deletions

Homozygous deletions are important genetic events as they can identify relevant genes within deleted regions as they, by definition, fully inactivate genes contained within them. Homozygous deletions of at least 100 kb in single cases were identified at *FAM46C* (1p), *TSPYL4* (6q), *PARK2* (6q), *TLR4* (9q), *RB1* (13q), *WWOX* (16q), *CDH1* (16q), keratin locus (17q), *GSK3A* and neighboring genes (19q), *UTX* (Xp), *CNKSR2* (Xp), and *HDHD1A* (Xp). Frequently occurring homozygous deletions were located at 1p32.3 (*FAF1*/*CDKN2C*), 11q (*BIRC2* and *BIRC3*), 14q (*TRAF3* and *AMN*), and 16q (*CYLD*). The majority of these genes have known relevance to myeloma biology, such as *CDKN2C* and *RB1* (cell-cycle regulation), *TRAF3*, *BIRC2*, *BIRC3* and *CYLD* (NF-κB regulation),[6-8] *WWOX* (apoptosis),[7,32] *GSK3A* (Wnt signaling),[14,33] and *CDH1* (frequently methylated).[34,35]

Pathways analysis of deregulated genes in myeloma

To determine the potential biologic significance of the CNAs, a pathway analysis of differentially expressed genes within the regions of interest, including homozygously deleted regions, was performed. Using the KEGG pathway analysis tool within DAVID[36] we identified the Wnt signaling pathway as being enriched ($P = .0026$), along with the Apoptosis pathway ($P = .046$). Genes belonging to the Wnt signaling pathway were *NFATC3*, *PPP3CC*, *FZD3*, *CTBP1*, *SIAH1*, *PSEN1*, *GSK3B*, *CSK22*, and *CSNK2B*. Those involved in apoptosis were *PPP3CC*, *BIRC2*, *TNFRSF10A*, *TNFRSF10B*, and *TRADD*. In addition, some of the genes may be more relevant to other pathways, such as *BIRC2* and *TRADD* with the NF-κB pathway. The genes identified in this analysis point toward several biologic networks relevant to myeloma biology. Cell-cycle regulation genes such as *CDKN2C*, *CDKN2A*, *CDKN2B*, *CDKN1B*, and *RB1*, are deleted and underexpressed and would be predicted to result in abnormalities of cell-cycle progression, as has been suggested by the role of cyclin D overexpression.

Wnt gene signaling is important in stem cell biology but in this context may be relevant to G_1/S deregulation via the impact of *GSK3A* directly onto the function of cyclin D1, whereas loss of negative regulators of the NF-κB pathway would facilitate a ligand-independent survival advantage for cells. *FAF1*, which has also been implicated in the negative regulation of NF-κB and FAS-associated cell death signaling and apoptosis pathways, may also be important. The other genes involved in apoptosis include *TNFRSF10A*/*D* potentially implicating TRAIL signaling deregulation in the immortalization of the myeloma clone. *TP53* abnormalities are also important in both clonal immortalization and survival after treatment and deletion/mutation of this gene are one of the most prognostically important acquired genetic alterations in myeloma.

Histone methylation or acetylation also plays a key role in the pathogenesis of myeloma. Of particular relevance to this process is *MMSET*, which is overexpressed due to the t(4;14) translocation.[37] *MMSET* is a histone methyltransferase and is thought to act as a transcriptional repressor through methylation of histone lysine residues.[38-40] In addition to *MMSET*, *UTX* has been shown to be deregulated in up to 10% of myeloma samples. *UTX* is a histone demethylase, performing the opposite function to *MMSET*, and is either mutated or deleted in myeloma resulting in histone methylation in the myeloma epigenome.[41] We have also identified *ANP32E* as a potential partner in the regulation of histones in myeloma. *ANP32E* is a histone acetyltransferase inhibitor (INHAT)[31] and is over-expressed in samples with gain of 1q. Overexpression of this gene would potentially result in increased histone methylation, therefore adding weight to the theory that

histone modification and epigenetics in myeloma are key to the pathogenesis of the disease.

The chromosomal alterations in presenting myeloma cells are diverse and include balanced and unbalanced translocations, UPD, deletions and gains of regions and whole chromosomes, and homozygous deletions of genes important in the pathogenesis of the disease. It is clear that although NF-κB has been implicated in the disease, it is not the sole contributing factor and we have shown other genes and pathways that have an important prognostic impact on the future treatment of myeloma patients.

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Authorship

Contribution: B.A.W. performed research, analyzed data, and wrote the paper; P.E.L., L.C., K.D.B., D.C.J., G.P.D., R.K.M.P., Z.J.K., and D.M.S. performed research and analyzed data; N.J.D., M.W.J., and D.G. analyzed data; W.M.G. collated clinical information; F.E.D. designed research; F.M.R. designed and performed research and analyzed data; and G.J.M. designed research and wrote the paper.

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