Integration of global SNP-based mapping and expression arrays reveals key regions, mechanisms, and genes important in the pathogenesis of multiple myeloma

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Multiple myeloma is characterized by genomic alterations frequently involving gains and losses of chromosomes. Single nucleotide polymorphism (SNP)–based mapping arrays allow the identification of copy number changes at the sub-megabase level and the identification of loss of heterozygosity (LOH) due to monosomy and uniparental disomy (UPD). We have found that SNP-based mapping array data and fluorescence in situ hybridization (FISH) copy number data correlated well, making the technique robust as a tool to investigate myeloma genomics. The most frequently identified alterations are located at 1p, 1q, 6q, 8p, 13, and 16q. LOH is found in these large regions and also in smaller regions throughout the genome with a median size of 1 Mb. We have identified that UPD is prevalent in myeloma and occurs through a number of mechanisms including mitotic nondisjunction and mitotic recombination. For the first time in myeloma, integration of mapping and expression data has allowed us to reduce the complexity of standard gene expression data and identify candidate genes important in both the transition from normal to monoclonal gammopathy of unknown significance (MGUS) to myeloma and in different subgroups within myeloma. We have documented these genes, providing a focus for further studies to identify and characterize those that are key in the pathogenesis of myeloma. (Blood. 2006;108: 1733-1743)

Introduction

The genome of myeloma plasma cells is characterized by marked instability comprising both complex numeric and structural abnormalities.[1] Conventional cytogenetic studies fail to identify these abnormalities in most cases due to the low proliferative index of myeloma plasma cells. However, interphase fluorescence in situ hybridization (FISH) enables the identification of an abnormality in up to 96% of cases.[2] Myeloma can be broadly categorized into 2 subgroups, hyperdiploid and non-hyperdiploid, based on the modal distribution of chromosome number.[3-5] Recurrent immunoglobulin gene chromosomal translocations, which are well described etiologic events in myeloma, are seen in approximately 40% of the total. However, the translocations are unevenly distributed between the 2 groups, being seen in over 85% of the non-hyperdiploid and in less than 30% of the hyperdiploid group, consistent with 2 differing pathways to malignancy.[3,4,6]

The recurrent chromosomal translocations occur as a result of physiologic class switch recombination, occur in the majority of clonal cells, and are found in a similar proportion of both monoclonal gammopathy of unknown significance (MGUS) and myeloma cases.[7-9] Late-stage clinical samples and human myeloma cell lines (HMCLs) are characterized by a number of pathologic events such as mutations of *TP53* and *RAS* and methylation of *CDKN2A*.[10-16] This is consistent with a multistep model of disease progression from MGUS through myeloma to extramedullary myeloma. However, the nature of the intermediate pathologic events responsible for the progression from MGUS to myeloma is not well described.

Interstitial deletion or gain of either whole or parts of chromosomal regions is a common mechanism of disease progression in both lymphoid and nonlymphoid tumors. The relevance of such mechanisms to myeloma is supported by prior observations from cytogenetic analysis. Various regions of chromosomal copy number change have been described including the deletion of 1p, 6q, 8p, 13q, 16q, 17p, and 22q and gains of 1q, 6p, 9q, 11q, 12q, 15q, 17q, and 19q (Table 1).[17-22] Of these copy number changes, the most widely recognized is del(13), which is present in approximately 30% to 55% of the total myeloma population.[7,23-26]

Alleles can also be lost through mechanisms other than loss of copy number, such as uniparental disomy (UPD). UPD arises via a number of mechanisms, including monosomic and trisomic rescue

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Table 1. Summary of chromosomal copy number changes identified by CC, FISH, and CGH

Chromosome	Lai et al[20] CC (% of 66 abnormal)*	Nilsson et al[22] CC (abnormal CC %)*	Cremer et al[18] FISH (%)	Liebisch et al[21] FISH (%)	Cigudosa et al[17] CGH (%)	Liebisch et al[21] CGH (%)	Gutierrez et al[19] CGH (% of 51 abnormal)*
Loss							
1	NA	1p (p21-22) (17)	ND	ND	NA	1p (p21) (13)	1p (10)
2	NA	NA	ND	ND	NA	NA	NA
4	NA	NA	ND	ND	NA	NA	NA
5	NA	NA	ND	ND	NA	NA	NA
6	NA	6q (q21-27) (11)	6q21 (16)	6q (21)	6q (q21) (13)	6q (15)	6q (10)
8	8 (20)	NA	8p12 (21)	ND	NA	NA8p (p21) (15)	8p (10)
10	10 (12)	NA	ND	ND	NA	NA	NA
12	12 (11)	NA	ND	ND	NA	NA	NA
13	13 (33)	13 (27)	13q14 (54)	13q (41)	13q (q14-21) (30)	13q (q14-21) (37)	13q (39)
14	14 (20)	14 (12)	ND	ND	NA	NA	14q (12)
15	15 (12)	NA	ND	ND	NA	NA	NA
16	NA	16 (14)	ND	ND	16 (17)	NA	16q (18)
17	NA	NA	17p13 (26)	ND	NA	NA	NA
20	20 (14)	NA	ND	ND	NA	NA	NA
22	NA	NA	22q11 (25)	ND	NA	NA	NA
X	X (23)	X (17)	ND	ND	NA	X (15)	NA
Y	Y (17)	Y (20% males)	ND	ND	NA	NA	Y (12% males)
Gain							
1	NA	1q (q10-44) (13)	1q21 (44)	1p (15)	NA	1q (q21-23, q25-31) (26)	1p (10%) 1q (45%)
2	2 (11)	NA	ND	ND	NA	NA	NA
3	3 (44)	3 (18)	ND	ND	NA	NA	3q (16)
4	NA	5 (20)	ND	ND	NA	NA	4q (10)
5	5 (26)	NA	ND	ND	NA	5 (11)	5q (24)
6	6 (11)	NA	ND	6q (15)	NA	6p (p21) (11)	6p (12)
7	7 (21)	7 (19)	ND	ND	NA	NA	7q (14)
8	NA	NA	ND	ND	NA	NA	8q (10)
9	9 (39)	9 (27)	9q34 (61)	ND	9q (10)	9q (q31qter) (15%) 9p (11%)	9q (24)
11	11 (27)	11 (20)	11q23 (57)	11q (42)	11q (20)	11q (q23) (15)	11q (22)
12	NA	NA	ND	ND	12q24 (10)	NA	12q (10)
15	15 (29)	15 (24)	15q22 (63)	ND	15q23 (10)	15 (11)	15q (22)
17	NA	NA	ND	17p (14)	17q22-24 (10)	NA	17q (10)
18	18 (17)	18 (10)	ND	ND	NA	18q (11)	NA
19	19 (35)	19 (22)	19q13 (61)	ND	19 (p) (30)	NA	19q (18)
21	21 (17)	21 (15)	ND	ND	NA	NA	NA
22	NA	NA	ND	ND	22q (10)	NA	22q (10)
X	NA	NA	ND	ND	NA	NA	Xq (10)

For Lai et al, n = 129; for Nilsson et al, n = 783; for Cremer et al, n = 81; for Liebisch et al FISH, n = 43; for Cigudosa et al, n = 25; for Liebisch et al CGH, n = 46; and for Gutierrez et al, n = 74.

CC indicates conventional cytogenetics; NA, not applicable; ND, not done.

*Only incidents greater than 10% included.

(in embryonic development), incomplete segregation of chromosomes, and mitotic recombination (Figure 1). Functional allelic loss occurs where one allele is deleted and the remaining dysfunctional allele is duplicated resulting in loss of heterozygosity (LOH) without loss of copy number.[27] UPD has been described in many cancers[28-31] including acute myeloid leukemia (AML), where the characteristic features are long runs of UPD often stretching from the middle of the chromosomal arm to the telomere.[27] The relevance of this mechanism to the etiology of AML has been confirmed by the demonstration of mutation within key AML-specific genes contained within the runs of UPD.

Comparative genomic hybridization (CGH), on normal metaphase spreads, has been used to examine the whole tumor cell genome for chromosomal copy number changes. Although multiple genomic aberrations can be identified using this methodology, the resolution is limited to approximately 10 to 20 Mb.[32] In contrast, array CGH using BAC clones arrayed onto a solid surface is more sensitive and is able to identify regions of chromosomal copy number change with a resolution as low as 1 Mb. The advent of single nucleotide polymorphism (SNP)–based technology, however, has increased this resolution further and can identify copy number changes at sub-megabase resolution. In addition, such SNP-based techniques have the advantage of not only identifying copy number changes but also identifying copy number-neutral LOH, such as UPD. SNP-based arrays consist of sets of oligonucleotides specific for polymorphisms in the genome. Although an oversimplification, each SNP has 2 different oligo sets, one for each allele, which when hybridized with sample DNA give a signal intensity relating to copy number and a SNP call referring to the allele in the sample, which can either be AA, BB, or the heterozygous call AB.

In this work, we describe the use of SNP-based mapping arrays to identify collaborating genetic events in a series of cytogenetically defined subgroups of myeloma consisting of cases with a t(4;14), t(11;14), and hyperdiploidy. We then use the data to define classes within the samples to drive clustering of expression array data and define genes, within regions of LOH, important in the pathogenesis of multiple myeloma.

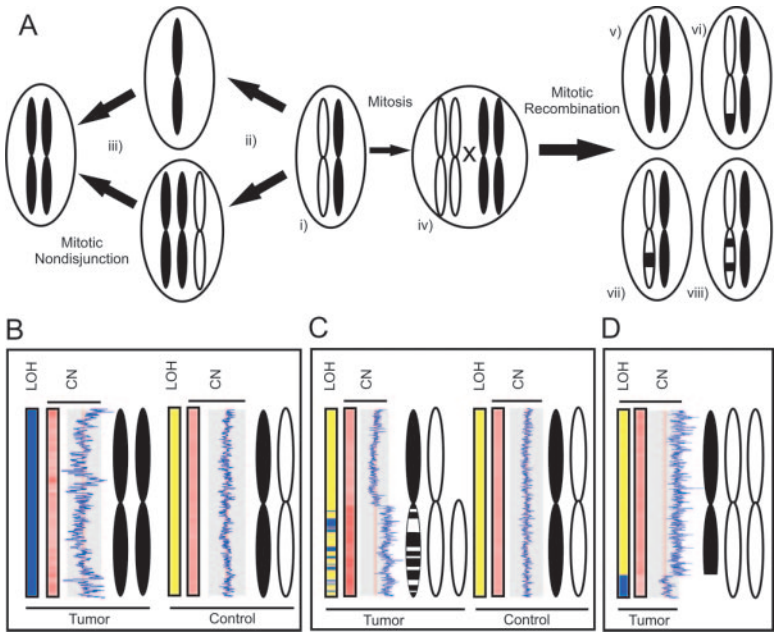

Figure 1. Mechanisms of uniparental disomy in myeloma. (A) In a normal cell (i) both parental alleles are present. During mitosis incomplete segregation of chromosomes can occur (ii) resulting in either monosomic or trisomic cells for a particular chromosome. UPD of a whole chromosome can then occur (iii) through mitotic nondisjunction, where the remaining chromosome is duplicated in the monosomic cell, or where the outnumbered allele is deleted in the trisomic cell. Alternatively, during mitosis (iv) recombination between chromatids can occur resulting in UPD of an arm of a chromosome (v) or part of an arm to the telomere (vi). If multiple recombination events occur during mitosis then interstitial regions of UPD can occur (vii-viii). (B) UPD in a myeloma sample occurring through mitotic nondisjunction on chromosome 12. (C) Uniparental trisomy occurring in myeloma by mitotic recombination and gain of chromosome 1q. (D) UPD occurring in myeloma by deletion of the telomere of chromosome 2. Regions of LOH are shown in blue, with heterozygous regions in yellow. Copy number is shown by both the red bar and the gray panel, where the blue line represents the copy number of the sample and the red line a copy number of 2 (with the edges of the gray panel indicating copy numbers of 1 [left] and 3 [right]). The sample is shown pictorially alongside.

Materials and methods

Sample preparation

Bone marrow aspirate samples were obtained after informed consent was given by patients entered into the MRC Myeloma IX trial. Patients were newly diagnosed with multiple myeloma and had not undergone any previous treatment. Plasma cells were selected as previously described,[33] to a purity of more than 90% using CD138 microbeads and magnetic-assisted cell sorting (Miltenyi Biotech, Bergisch Gladbach, Germany). Selected cells were split for analysis by FISH and for extraction of RNA and DNA. FISH was performed using standard approaches aimed at identifying translocation partners (t(4;14), n = 7 and t(11;14), n = 12) and hyperdiploid status (n = 11) by examining chromosomes 3, 4, 5, 7, 9, 11, 13, 14, 15, and 17 using previously described probes.[2] Hyperdiploidy was defined primarily on the results of chromosomes 5, 9, and 15[5] but modified by the results from other probes used (manuscript in preparation). Cytogenetic analysis was attempted on 25 of 30 samples and gave abnormal results in 10 (44% attempted, 33% total). Cells for RNA and DNA extraction were frozen in RLT buffer (Qiagen, Valencia, CA) immediately after selection. RNA and DNA were extracted using commercially available kits (RNA/DNA mini kit or Allprep kit, Qiagen) according to the manufacturer's instructions. Matched germline DNA from 12 patients was also extracted from peripheral white blood cells, taken at time of entry into the study, using the Flexigene kit (Qiagen). RNA and DNA quality and quantity was determined using a 2100 Bio-analyser (Agilent, Palo Alto, CA) and an ND-1000 Spectrophotometer (Nano-Drop Technologies, Wilmington, DE), respectively.

Genome mapping and expression analysis

DNA (250 ng) was prepared using the GeneChip mapping assay protocol (Affymetrix, Santa Clara, CA) for hybridization to GeneChip Mapping 50K Xba 240 Arrays according to the manufacturer's instructions. For expression arrays 100 ng total RNA was amplified using a 2-cycle target labeling kit (Affymetrix) per the manufacturer's instructions. Amplified cRNA (15 μg) was hybridized to Human Genome U133 Plus 2.0 arrays as per the protocol. Arrays were washed on an Affymetrix Fluidics Station 450 and scanned using a GeneChip Scanner 3000. (Raw data can be accessed at www.icr.ac.uk.)

Copy number

SNP genotypes were obtained using Affymetrix GCOS software (version 1.3) to obtain raw feature intensity, which was then processed using Affymetrix GDAS software (version 3.0.2) to derive SNP genotypes. Raw copy number and LOH analysis data for individual samples was generated using Affymetrix CNAT software (version 2.2.0.1). Multiple samples were analyzed together using output from GCOS and GDAS using dChipSNP.[34] The 12 control samples were assigned a copy number of 2 and used as a reference set to calculate copy number in tumor samples. Median smoothing with a window size of 11 was used to infer copy number along each chromosome. All results were verified using outputs from CNAG.[35]

LOH analysis

LOH analysis was performed with dChipSNP using a Hidden Markov Model to infer the probability of LOH based on the observed LOH scores (based on the 12 paired control/tumor samples) or genotype calls alone (from tumor samples without paired normal) using an average heterozygosity rate of 0.27.

Integration of expression and SNP mapping array data

The samples were grouped into 2 classes based on presence or absence of LOH or gain at the region of interest. Supervised hierarchical clustering was then performed using dChip, based on these classes, to determine genes that are differentially expressed between the classes. Samples without LOH or gain were used as the baseline (B) and compared to those with LOH or chromosomal gain (E). Comparison criteria used were lower bound-fold change E/B greater than 1.1 or B/E greater than 1.1, mean difference E-B greater than 10 or B-E greater than 10, *t* test P less than .05. Rather than define a global gene expression pattern for the classes, we were interested in the expression of the genes located at that region of interest. Gene lists were generated containing only genes within the region with LOH or gain.

Quantitative short tandem repeat typing

Copy number and presence of alleles were checked on one sample using the PowerPlex 16 System (Promega, Madison, WI). DNA (1 ng) was prepared according to the manufacturer's instructions and run on a 3130xl capillary sequencer (ABI, Foster City, CA).

Results

This study used 30 samples from newly diagnosed, previously untreated patients with multiple myeloma, with 12 paired peripheral blood controls. A number of parameters were monitored during sample processing to determine sample quality and final quality of the SNP array data, the final arbiter of data quality being SNP call rate. Median SNP call rate per array in the 30 tumor samples was 94.89% (range, 79.76%-98.34%) and in the 12 control samples 97.92% (range, 95.70%-98.70%). Accuracy of SNP calls was validated using the presence of heterozygous calls on the X chromosome of male patients in normal and tumor samples. In 16 male patients there was a median of 9 heterozygous calls (range, 2-41) of 1204 SNPs (0.75%) on the X chromosome indicating 99.25% accuracy.

FISH data compared favorably with SNP array data, with respect to copy number in the samples, with 162 of 174 FISH tests matching SNP copy number data. However, 3 samples were identified as being near tetraploid by FISH, but were classified as diploid by SNP array. This discrepancy has also been found with CGH and array CGH.

Analysis of the 12 control samples using dChip revealed the presence of both large and small regions of inferred LOH throughout the genome. Most of these were small, consisting of 20 to 40 SNPs with a seemingly random genomic distribution, but in 2 cases there were large regions of UPD, copy number-independent LOH, in both control and tumor samples involving whole arms of chromosomes. The regions involved chromosome regions 4q, 8p, and 14q and the pericentric region of chromosome 19.

UPD

Paired tumor/control samples are able to identify LOH by true allelic imbalance or acquired UPD. Multiple regions of UPD were identified in the myeloma plasma cells ranging in size from 677 kb to whole chromosomes and were interspersed throughout the genome. The median number of regions of UPD was 3 per sample (range, 0-19). Regions of UPD were more frequent on the larger chromosomes, with the median number of regions of UPD on the first 7 chromosomes being 11 in the 30 samples compared to a median of 5 per chromosome in the 30 samples when looking at all of the chromosomes. The mean number of regions of UPD was 2, 5, and 7 in the t(11;14), hyperdiploid, and t(4;14) cases, respectively. The regions of UPD were generally small and rarely extended to the telomere of the chromosome, indicating that they probably arose through multiple mitotic recombination events. There were 4 incidences of UPD to the telomere of the chromosome (arising from one recombination event) and 2 of these were on chromosome 1. There was one example of complete chromosomal UPD (chromosome 12) of 30 samples, which probably arose through mitotic nondisjunction (Figure 1).

LOH

Identification of regions of LOH using dChip showed that there was a median number of 14 regions of LOH per patient (range, 4-30) with a median size of 1026 kb. We focused on regions that were present in at least 10% of the samples (ie, 3 cases) and identified 117 separate areas of LOH (Figure 2; Table S1, available on the Blood website; see the Supplemental Tables link at the top of the online article).

The regions of chromosomal change are summarized in Figure 2 and Table 2. The average number of regions of copy number change ranges from zero to numerous regions per chromosome depending on the sample. The size of regions ranged from 22 kb containing no transcripts to entire chromosomes. In our samples we found loss of 1p (23%), 6q (13%), 8p (30%), 13q (53%), and 16q (20%) and in female samples loss of chromosome X (57%). These data tally well with previously reported data sets (Table 1), with the exception of loss of the X chromosome, which is found at a higher rate in this study.[20-22,36] We found gains of 1q (36%) and 6p (16%) and, as expected, gains of odd numbered chromosomes in hyperdiploid samples.

All 7 cases with the t(4;14) translocation exhibited numerous regions of chromosomal copy number change indicating considerable genomic instability. A similar feature was seen in the hyperdiploid cases. Three of the t(4;14) cases had gains of chromosomes that met the criteria for hyperdiploid status and, in addition, this group appeared to be associated with the gain of the whole arm of 1q (5 of 7 cases), trisomy of chromosomes 15 (4 cases), 19 (3 cases), and 3, 5, and 21 (2 cases) and deletion of chromosome 13 (all cases).

In contrast to the t(4;14) cases, the genome of 12 cases with the t(11;14) was more stable with no change in copy number seen in 2 of 12 t(11;14) samples, none meeting the criteria for hyperdiploidy and only 6 of 12 cases having chromosome 13 deletion. An additional finding present in these samples was that 3 of the 12 t(11;14) samples had deletion of the whole arm of 16q, whereas no examples of this were identified in the t(4;14) samples.

We compared the results of FISH analysis with the SNP array data in the 11 cases categorized as hyperdiploid by FISH. Copy number analysis showed that chromosomes 3, 5, 7, 9, 11, 15, 19, and 21 had increased copy number more frequently than the other chromosomes. Chromosome 19 was amplified in all cases followed by chromosomes 15, 9, 11, 3, 5, and finally chromosome 7 being amplified in 5 samples. Using the same approach we examined the nontrisomic chromosomes. Chromosomes 1, 6, 8, and 13 showed changes in copy number in more than 2 (range, 3-7) of the 11 hyperdiploid samples. There was loss of 8p in 6 hyperdiploid samples, of which one sample also had gain of 8q and there was monosomy of chromosome 13 in 3 hyperdiploid samples. There was gain of 6p in 3 samples, gain of 1q in 5 samples, and deletion of 1p12-21.2 in one sample.

Chromosome 1

Copy number alterations or translocations involving chromosome 1 have been well described in myeloma. Our study confirms frequent deletions of 1p12-p31.1 (7 of 30), with a minimally deleted region between 1p12 and 1p21.1. Two additional samples also had complete UPD of 1p. Using these samples in supervised hierarchical clustering of gene expression data resulted in 2254 genes that were differentially expressed, of which 52 were located in 1p12-p21.1. Fifty-one of these genes were underexpressed in the samples with deletion and one was overexpressed in the samples with deletion.

Amplifications of the whole arm of 1q (11 of 30) were identified, including one case with apparent regions of uniparental trisomy (UPT) of 1q23.3-q24.1, 1q31.1-q31.2, 1q41, and 1q43 arising through mitotic recombination and gain of 1q (Figure 1C). We could not identify a recurrent minimally amplified region on 1q. Gene expression profiling of the 1q amplified group revealed 1306 genes differentially expressed, of which 102 were located on 1q (Table S2). Eight of these genes were underexpressed in the 1q amplified group, whereas 94 were overexpressed. The genes

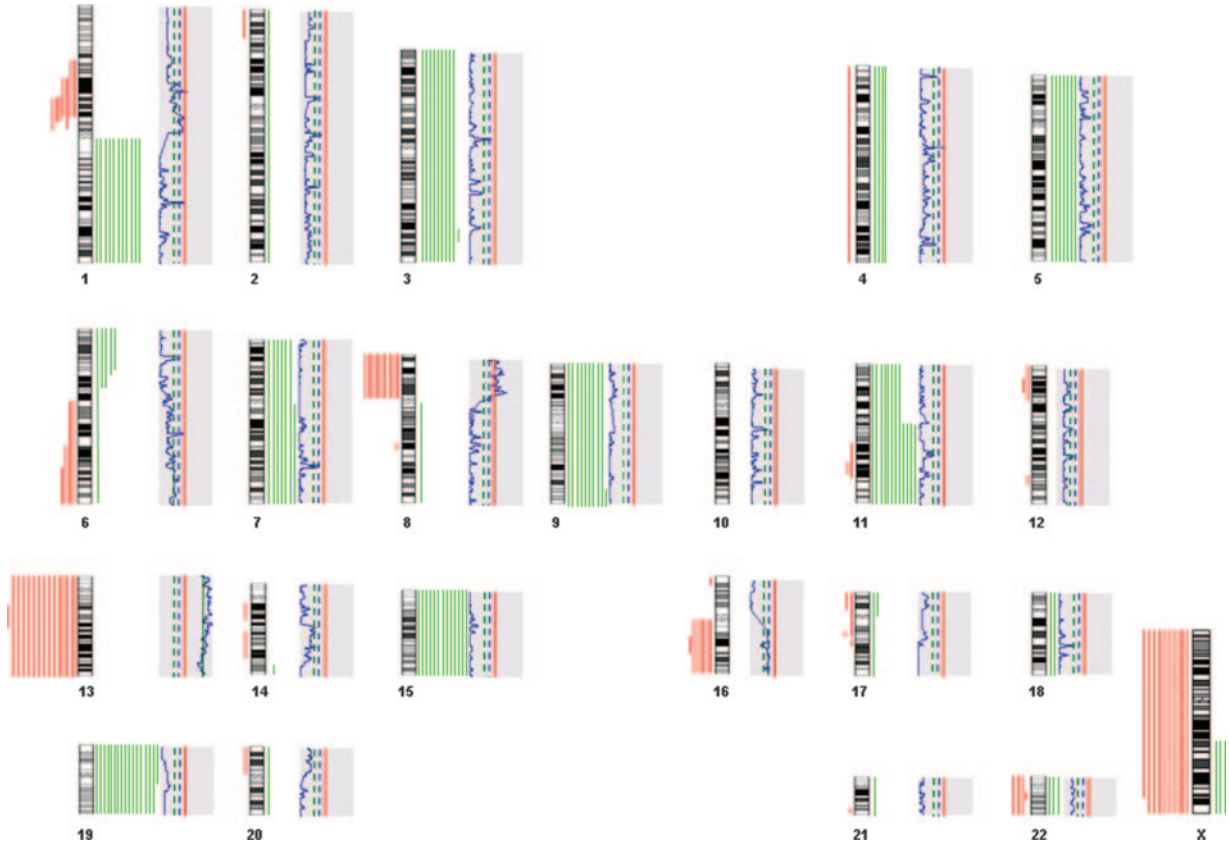

Figure 2. Regions of LOH and chromosomal gain and loss in myeloma samples, as determined by SNP array. Chromosomes are numbered under the ideogram, with regions of gain indicated to the right of the ideogram in green and regions of deletion to the left of the ideogram in red, for each sample. LOH is shown in the gray panel to the right of the ideogram, where the blue line indicates the number of samples with LOH at that point on the chromosome. The dashed green line indicates the 3-sample mark, the blue dashed line the 5-sample mark, and the red line the 7-sample mark.

identified are not located in any particular region of 1q. Previous studies have suggested that either *CKS1B*, *BCL-9*, or *PDZK1* may be the key gene overexpressed in 1q amplified cases.[37-41] In our series, there was no statistical difference in expression of any of these 3 genes between samples with and without gain of 1q.

Chromosome 6q

Deletion of the whole of 6q was seen in 2 samples and in a further 2 samples deletion of 6q23.2-6qter was seen. These were distributed across all 3 subgroups. Supervised hierarchical clustering of the 4 cases with the common deleted region 6q23.2-6qter identified 963 genes differentially expressed between those with the deletion and without. Of these, 30 genes were located in 6q23-qter, all of which were underexpressed in cases with the deletion. Two of these genes have been identified as potential tumor suppressor genes in other malignancies, *PERP* and *SOD2*.[42,43] Both were also underexpressed in 12 and 16 nondeleted cases, respectively.

Chromosome 8p

LOH on chromosome 8p was seen in 33% (10 of 30) of samples. UPD was seen in one sample on chromosome 8p, but was also present in the matched peripheral blood DNA control and so has not been included in these analyses. Deletion of the whole arm of 8p was seen in 5 samples, of which 3 are hyperdiploid cases, one case had a t(11;14) and one case a t(4;14) as shown in Figure 3. An additional 4 samples had more than 50% deletion of 8p, with either the telomere or centromere region of 8p remaining intact (3 hyperdiploid and one t(4;14)). Six other samples had smaller regions of deletions across 8p (3 hyperdiploid, one t(4;14), 2 t(11;14)). Fourteen samples were normal for 8p (4 hyperdiploid, 2 t(4;14), 8 t(11;14)). The nature and extent of the regions of LOH are shown in Figure 3. We could not identify a significant difference in distribution of 8p abnormalities between the different cytogenetic subgroups studied.

By performing supervised clustering between samples with and without LOH on 8p we identified 46 genes with significant expression differences (Table S2). All of the genes with differential expression identified on 8p were down-regulated in the samples with LOH. Transcripts for *ADAM28*, *STC1*, *BLK* (tyrosine kinase), *MTUS1* (tumor suppressor), and a hypothetical protein had the largest fold changes in expression. In this study we have identified many genes that are down-regulated on 8p in myeloma samples, which are potential tumor suppressor genes.

Chromosome 13

Deletions of chromosome 13 were seen in 53% (16 of 30) of the cases and there was 100% correlation between FISH and SNP array results. One case had deletion of only 13q13.3-q21.3, which encompasses *RB1* and *D13S319* (the probes used for FISH), whereas the other cases had deletion of the whole of chromosome 13. Gene expression analysis data for chromosome 13 can be found in Table S2.

One case was identified that had a deletion by FISH analysis and showed loss of 13q by copy number but it did not have LOH of 13q, except for 1.9 Mb at 13q21.31 (Figure 4A). This case

Table 2. Summary of copy number change in 30 myeloma samples, split into the three groups: t(4;14), t(11;14), and HRD

	Gain				Loss			
Chromosome	t(4;14)	t(11;14)	HRD	χ^2 (P)	t(4;14)	t(11;14)	HRD	χ^2 (P)
1	+1q [5]	+1q [1]	+1q [5]	8.15 (.025)	–1p [5]	−1p [1]	−1p [1]	11.1 (.01)
2	—	—	+2 [1]	1.78 (NS)	—	−2p [1]	—	1.46 (NS)
3	+3 [2], +3q [1]	—	+3 [7]	10.8 (.01)	—	—	—	—
4	+4 [1]	+4 [1]	+4 [1]	0.19 (NS)	−4 [1]	—	—	3.4 (NS)
5	+5 [2]	—	+5 [5]	6.7 (.05)	—	—	—	—
6	+6 [1], +6p [1]	—	+6p [3]	4.0 (NS)	−6q [1]	−6q [2]	−6q [1]	0.29 (NS)
7	+7 [1], +7q [1]	—	+7 [5]	6.7 (.05)	—	—	—	—
8	—	—	+8q [1]	1.78 (NS)	−8p [2]	−8p [1], −8q [1]	−8p [6]	5.84 (NS)
9	+9 [1]	+9q [1]	+9 [8]	12.2 (.01)	−9p [1]	—	—	3.39 (NS)
10	—	—	—	—	—	—	—	—
11	+11 [1]	+11q [3]	+11 [7], +11q [1]	7.9 (.025)	−11q [1]	—	—	3.39 (NS)
12	—	—	—	—	−12p [2], −12q [1]	—	—	7 (.05)
13	—	—	—	—	−13 [7]	−13 [6]	−13 [2]	11.45 (.01)
14	—	—	—	—	−14q [2]	—	—	7.0 (.05)
15	+15 [4]	—	+15 [9]	16.35 (.001)	—	—	—	—
16	—	—	—	—	—	−16p [1], −16q [4]	−16q [2]	3.0 (NS)
17	—	—	+17 [1], +17p [1]	3.7 (.2)	−17q [1]	−17p [2]	−17q [1]	3.2 (NS)
18	—	+18 [1]	+18 [1]	0.67 (NS)	—	—	—	—
19	+19 [3]	+19 [3]	+19 [11]	13.85 (.01)	—	—	—	—
20	—	—	+20 [1]	1.7 (NS)	−20p [1]	—	—	3.39 (NS)
21	+21 [1]	—	—	3.39 (NS)	−21q [1]	—	—	3.39 (NS)
22	—	—	—	—	−22q [2]	−22q [1]	−22q [1]	1.87 (NS)
X	+Xq [1]	+Xq [1]	+Xq [1]	0.18 (NS)	−X [3]	−X [5]	−X [2]	1.79 (NS)

Only large regions of chromosomal gain and loss are indicated, with numbers of cases in square brackets. χ^2 performed: for significance at $P < .05$ level, χ^2 result should be greater than 5.99. For t(4;14), n = 7; for t(11;14), n = 12; and for HRD, n = 11.

HRD indicates hyperdiploid; —, no change; NS, not significant.

was also studied by short tandem repeat-polymerase chain reaction (STR-PCR) to determine the heterozygosity status of chromosome 13 (Figure 4B). This confirmed that the sample was heterozygous, but it had half the amount of each allele compared to the control sample.

Clustering the LOH data using dChip grouped all of the del(13) cases together allowing us to look for associated copy number change, which could explain the clinical findings associated with this group. The most obvious associated genetic lesion was loss of the X chromosome in the female patients. In fact, all female patients with del(13) also had monosomy or LOH (through deletion and subsequent duplication) of the X chromosome (Figure 4C).

Chromosome 16q

We identified deletion of the whole of 16q in 5 of 30 cases but no minimally deleted region was identified because the whole arm was hemizygous in all 5 cases. Gene expression profiling of those cases with 16q deletion compared with those without the deletion identified 1121 genes with differential expression in 16q deleted cases of which 60 were located on chromosome 16q, all of which were underexpressed (Table S2). These genes include the putative tumor suppressor genes *CYLD* and *WWOX*, which has been previously implicated in myeloma pathogenesis.[44,45] We examined the pattern of expression of *WWOX* in nondeleted cases and found that it is also underexpressed in 10 of 25 cases lacking a deletion suggesting that it may be inactivated by mechanisms other than deletion.

Integration of regions of LOH with genes important in myeloma pathogenesis

We have taken the consistent regions of LOH that occur in more than 10% of the total (Table S1) and documented the transcripts located within them, which identified 3041 genes (data not shown). Although this reduces the number of potential candidate genes for further analysis, it remains difficult to select candidate genes from such a list. To reduce this list further and to improve the selection process we compared this list to a list previously identified by our group as being important in the progression of normal through MGUS to myeloma plasma cells,[33] which identified 47 genes (Table 3). Twenty-three were identified when normal and MGUS plasma cells were compared and 16 of these are down-regulated in MGUS plasma cells. Thirty-eight genes were identified when normal and myeloma plasma cells were compared and 26 of these are down-regulated in myeloma plasma cells. Five of 8 genes were down-regulated when MGUS and myeloma plasma cells were compared (Table 3). The genes identified map to the major regions of interest, namely, 1p, 6q, 8p, 13, 16q, and 17p. In addition, the regions 14q22.1-24.1, 20p12.1-13, and 22q12.1-13.31 were identified as having at least 4 genes with a change in expression between normal, MGUS, and multiple myeloma plasma cells.

Discussion

With the implementation of high-density SNP arrays, it is now possible to genotype the whole human genome with a mapping resolution of less than 8.5 kb using 50K arrays. Thus, the SNP array approach offers an opportunity to analyze both copy number abnormalities and LOH simultaneously. Combining this information with data obtained from expression array data from the same samples provides a powerful way of identifying pathologically relevant genes hitherto impossible to identify. In addition to these data, the SNP arrays can give novel information about the mechanism by which the copy number changes in myeloma arise.

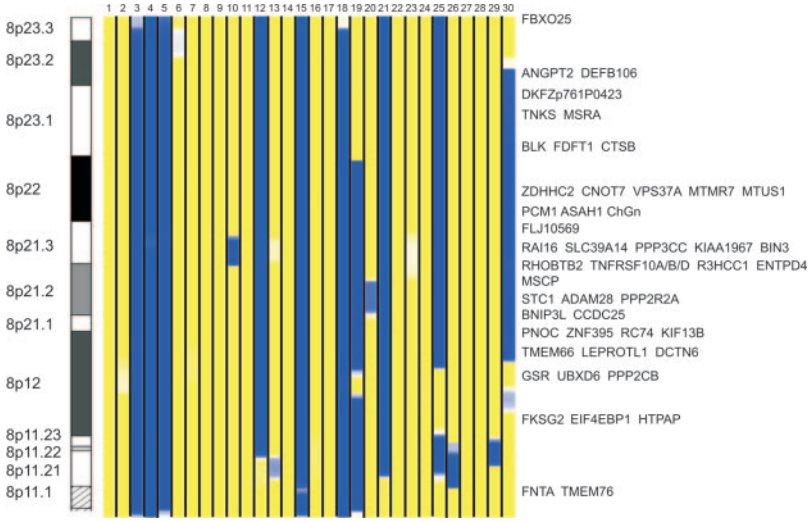

Figure 3. Regions of LOH on chromosome 8p can be used to drive supervised analysis of expression analysis to identify differentially expressed genes. Thirty samples (vertical lanes) with regions of LOH (blue) or heterozygosity (yellow) were used to identify which genes on 8p are differentially expressed between those samples with LOH and those without.

UPD, which describes copy number-neutral LOH, is a well-described mechanism in autosomal disorders where gene silencing of developmentally important genes leads to abnormalities without a change in copy number.[46] This mechanism has been shown to be important in cancer biology where it can lead to tumor suppressor gene inactivation.[47,48] Inactivation of one allele, by methylation or mutation, precedes loss of a functional allele and subsequent duplication of the inactive allele. We found UPD interspersed throughout the genome of myeloma plasma cells, but these regions tended to be small and probably arose through multiple mitotic recombinational events, unlike the situation in AML where large regions of UPD are found that arise from one mitotic recombinational event. However, we did identify one case of complete chromosomal UPD, which arises through mitotic nondisjunction. There were also 2 cases in which UPD was found in germline DNA, indicating the need for control DNA to distinguish acquired UPD from constitutive UPD. The identification of UPD in multiple myeloma gives rise to the possibility of identifying genes that are down-regulated, through epigenetic mechanisms such as methylation or deacetylation or through mutation, but retain a diploid copy number.

SNP array data can also give insight into the way that interstitial deletions arise. In particular, it can distinguish whether they occur during a single catastrophic event or if they arise continuously. In this study we identified one very informative case with a clonal deletion of chromosome 13. This case had loss of a copy of chromosome 13 by FISH, had a loss of 13q by SNP array, but did not have LOH of 13q. The only reasonable explanation for this is that multiple clones were present with different alleles of chromosome 13 deleted, suggesting that there may be ongoing deletion events at this site.

Recurrent chromosomal copy number changes occurring in myeloma are summarized in Table 1. This study has substantially added to these data showing both the presence of and narrowing down the extent of the lesions. These include 1p12-p31.1, 6q, 8p, 16q, and 22q12.1. It has also extended prior observations defining new regions of recurrent interstitial deletion that can be verified by LOH analysis. The definition of increased copy number is more difficult because there is not a secondary detection mechanism built into the analysis. It is possible to detect amplification of signal in single SNPs through to gains of whole chromosomes, although any small regions of gain need to be verified by other methods.

Changes of copy number on chromosome 1 are one of the most frequently reported copy number changes seen in myeloma. Amplification of 1q21 has been associated with poor prognosis in a number of studies implying that the region contains a critical gene

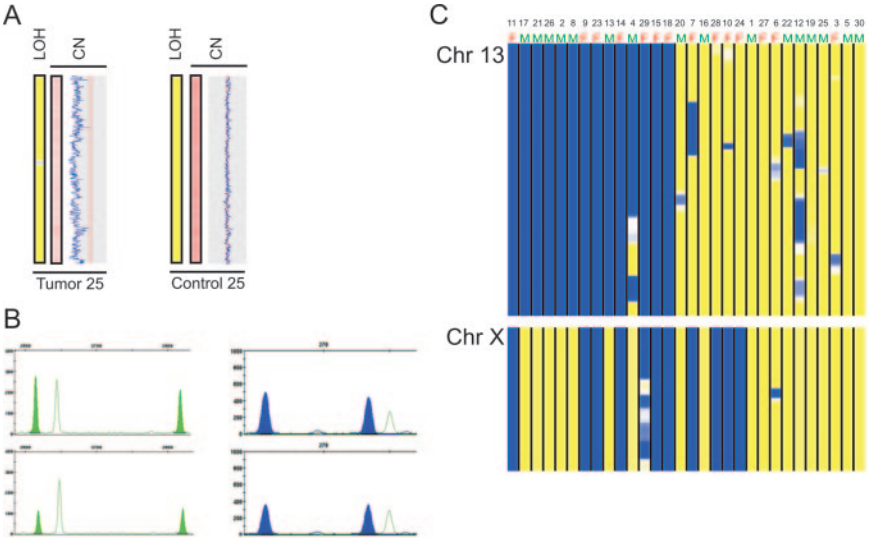

Figure 4. Analysis of chromosome 13 in myeloma samples. (A) Tumor sample 25 has monosomy of chromosome 13 without LOH, compared to the matched control. Figure 1 provides a description. (B) Quantitative analysis of short terminal repeats using the Powerplex 16 System shows that tumor sample 25 (bottom panels) has half the amount of both alleles of marker *D13S317* (green peaks) on chromosome 13 compared to the matched control (top panels). Analysis of other chromosomes, including chromosome 21 (marker *D21S11*, blue peaks), showed matching amounts of both alleles. Internal markers are shown as clear peaks. (C) LOH profiles of 30 myeloma samples, clustered according to LOH frequency by dChip, show that female samples with del(13) always have LOH of chromosome X. Top panel, chromosome 13; bottom panel, chromosome X. Regions of LOH are shown in blue and regions of heterozygosity in yellow. Sex of samples indicated at the top of each lane: M indicates male and F, female. Male samples do not show LOH (blue) on chromosome X due to homozygosity occurring naturally.

Table 3. Genes differentially expressed between normal, MGUS, and myeloma samples that are in regions of LOH present in 10% of samples as identified in Table S1

Chromosome	HGNC symbol	Description	NCBI accession no.	Normal vs. MGUS		Normal vs. MM		MGUS vs. MM	
				Fold change	*P*	Fold change	*P*	Fold change	*P*
1p13.3	*KCND3*	Potassium voltage-gated channel, Shal-related subfamily, member 3	AF048713	1.99	.003	1.74	.002	—	—
1p13-p12	*HMGCS2*	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 2 (mitochondrial)	X83618	—	—	−2.22	.036	−1.52	.032
1p31.1	*ACADM*	Acyl-coenzyme A dehydrogenase, C-4 to C-12 straight chain	M91432	—	—	—	—	−1.8	.040
4p16.1	*WDR1*	WD repeat domain 1	AL050108	—	—	—	—	−2.07	.009
5q31	*SEPP1*	Selenoprotein P, plasma, 1	Z11793	−6.2	.027	−10.04	.021	—	—
6p21.33	*HIST1H2BK*	Histone 1, H2bk	AJ223352	−3.24	.007	—	—	2.14	.020
6p22-p21.3	*HIST1H1B*	Histone 1, H1b	Z98744	—	—	2.82	.020	—	—
6q12	*PTP4A1*	Protein tyrosine phosphatase type IVA, member 1	U48296	−2.68	.031	−3.2	.021	—	—
6q22-q23	*MYB*	v-myb myeloblastosis viral oncogene homolog (avian)	U22376	2.16	.028	2.53	.005	—	—
6q25.1	*ESR1*	Estrogen receptor 1	X03635	—	—	1.75	.035	—	—
6q25-q27	*WTAP*	Wilms tumor 1 associated protein	D14661	—	—	−3.06	.026	—	—
8p12	*TMEM66*	Transmembrane protein 66	W26659	—	—	−2.25	.007	—	—
8p21.3	*DOK2*	Docking protein 2, 56 kDa	AF034970	—	—	4.07	.009	—	—
8p21.3	*GFRA2*	GDNF family receptor α 2	U97145	−3.22	.047	—	—	—	—
8p22-21.3	*ASAH1*	*N*-acylsphingosine amidohydrolase (acid ceramidase) 1	U70063	—	—	−2.72	.018	—	—
12p13.2	*GABARAPL1*	GABA(A) receptor-associated protein-like 1	W28281	—	—	−3.13	.043	—	—
12q23.3	*CKAP4*	Cytoskeleton-associated protein 4	X69910	−2.45	.029	−2.47	.028	—	—
13q12	*HMGB1*	High-mobility group box 1	D63874	—	—	−3.15	.030	—	—
13q12.1	*SAP18*	Sin3-associated polypeptide, 18 kDa	U96915	—	—	−4.41	.042	—	—
13q12.3-q1 3.1	*GTF3A*	General transcription factor IIIA	D32257	−2.81	.045	−3.2	.036	—	—
13q12-q13	*CG018*	Hypothetical gene CG018	AL049786	1.74	.011	—	—	—	—
13q12-q14	*TPT1*	Tumor protein, translationally controlled 1	X16064	—	—	−1.55	<.001	—	—
13q14.11	*MRPS31*	Mitochondrial ribosomal protein S31	Z68747	—	—	−3.21	.010	—	—
13q31.2-q3 2.3	*STK24*	Serine/threonine kinase 24 (STE20 homolog, yeast)	AF024636	−3.31	.004	−4.57	.004	—	—
13q32	*ZIC2*	Zic family member 2 (odd-paired homolog, *Drosophila*)	AF104902	—	—	1.31	.008	—	—
13q32.3	*TM9SF2*	Transmembrane 9 superfamily member 2	U81006	−3.23	.020	−4.28	.013	—	—
13q34	*ATP11A*	ATPase, class VI, type 11A	AB028944	2.34	.001	1.96	.003	—	—
13q34	*LAMP1*	Lysosomal-associated membrane protein 1	J04182	−2.9	.013	−4.47	.008	—	—
14q22-q24	*ZFP36L1*	Zinc finger protein 36, C3H type-like 1	X79067	−5.6	.006	−7.45	.004	—	—
14q24.1	*ACTN1*	Actinin, α 1	X15804	—	—	—	—	−23.19	.046
16q12.2-q13	*HERPUD1*	Endoplasmic reticulum stress-inducible, ubiquitin-like domain member 1	D14695	−1.99	.013	−2.94	<.001	—	—
16q13	*CNGB1*	Cyclic nucleotide gated channel β 1	U18945	2.92	<.001	2.84	.002	—	—
16q22.3q24.1	*GABARAPL2*	GABA(A) receptor-associated protein-like 2	AI565760	−2.44	.042	−2.92	.027	—	—
16q22-q23	*MAF*	v-maf musculoaponeurotic fibrosarcoma oncogene homolog (avian)	AF055376	—	—	−9.81	.049	—	—
16q24.2	*MAP1LC3B*	Microtubule-associated protein 1 light chain 3 β	W28807	−2.12	.031	−3.53	.008	—	—
16q24.3	*RPL13*	Ribosomal protein L13	X64707	—	—	—	—	1.5	.043
17p13.1	*GABARAP*	GABA(A) receptor-associated protein	AF044671	−2.52	.031	−3.26	.020	—	—
17p13.1	*SENP3*	SUMO1/sentrin/SMT3 specific protease 3	AL050283	—	—	2.04	.041	—	—
17p13.1	*CD68*	Serine/threonine kinase 17b (apoptosis-inducing)	AA203487	—	—	—	—	−2.02	.009
20p12	*BMP2*	Bone morphogenetic protein 2	M22489	—	—	85.74	.002	10.71	.039
20p12.1	*DSTN*	Destrin (actin depolymerizing factor)	S65738	—	—	−2.54	.005	—	—
20p13	*SOX12*	SRY (sex determining region Y)-box 12	U35612	1.62	.017	1.98	.004	—	—
20pter-p12	*PRNP*	Prion protein	U29185	−3.86	.006	−5.12	.005	—	—
22q12.1	*TPST2*	Tyrosylprotein sulfotransferase 2	AF049891	−4.02	.014	−6.72	.011	—	—
22q12.1	*XBP1*	X-box binding protein 1	Z93930	—	—	−1.72	.002	—	—
22q13.1	*TOM1*	Target of myb1 (chicken)	AJ006973	3.07	.009	—	—	—	—
22q13.31	*KIAA0153*	KIAA0153 protein	D63487	—	—	1.72	.006	—	—

MM indicates multiple myeloma; —, gene not found in this comparison.

important in the pathogenesis of myeloma.[37,40] Two candidate genes, *CKS1B* and *BCL9*, have been suggested to be those crucially deregulated. *CKS1B* has been reported to increase in expression with increases in copy number. However, it has been suggested that the increase in copy number correlates with late stage clinical disease following progression and so may not be evident in these presentation cases.[49]

The short arm of chromosome 8 is a frequent target of genetic alteration in a wide variety of human cancers including prostate, colorectal, breast, head and neck, and bladder carcinomas.[50-53] LOH of 8p in these cancers is often associated with a more aggressive tumor phenotype possibly indicating the presence of an important tumor suppressor gene.[54-56] Several genes on 8p have been examined including *RHOBTB2*, *LZTS1*, *TRAIL-R1* (*TNFSFR10A*), *TRAIL-R2* (*TNFSFR10B*), *DLC1*, *HTPAP*, *SFRP1*, *PCM1*, and *DUSP4*.[57-61] Our analysis identified a number of these genes as being down-regulated in samples with LOH. Interestingly, of the 46 genes we identified as being down-regulated in samples with LOH, 21 were also identified as being down-regulated in breast cancer samples with LOH of 8p.[57] This may give us a better insight into the genes that may be responsible for tumor progression in general and those that may be specific to myeloma.

Chromosome 13 is an important prognostic factor in myeloma and we have recently described the importance of FISH-based and cytogenetically detected deletions. In this analysis cytogenetically detectable deletions were shown to be responsible for the adverse prognosis associated with this lesion. Almost all cases with t(4;14) are associated with del(13), which may explain the poor prognosis of this cytogenetic subgroup. If del(13) were associated with poor prognosis it would be reasonable to identify a specific and recurrent minimally altered region (MAR). We could not define such a MAR at 13q, within which a single pathogenic gene could be identified, nor was correlation with expression levels of *RB1* seen. In agreement with our data, other groups have been unable to define a MAR on chromosome 13.[62-64]

The association of del(13) with another genetic lesion could explain its prognostic value. In this study, there was a strong association between del(13) and monosomy of chromosome X in female samples. No loss of chromosome X was found in male samples. Loss of X has previously been reported in 42% of female cases.[36] We did not find a firm correlation between del(13) and either deletion of 17p or alteration in *TP53* expression level.

In this study we provide evidence for the loss of expression of a gene, *WWOX*, located on chromosome 16 as being important in the pathogenesis of myeloma. This gene has been cloned as part of a recurrent switch region translocation and in this study we define deletion of 16q as being a further associated lesion. Loss of 16q is ubiquitously associated with loss of expression of *WWOX* but the latter is also seen in cases lacking LOH, consistent with its inactivation by other mechanisms. *WWOX* has been identified as a tumor suppressor gene and reduced expression of *WWOX* has been implicated in breast, lung, and bladder cancers through hypermethylation.[44,65] Several aberrant, nonfunctional transcripts of *WWOX* have been found, but point mutations occur infrequently.[44,66,67] These data support our hypothesis for multiple mechanisms of *WWOX* inactivation in myeloma.

In this analysis we have identified a number of novel recurrent regions of LOH and have concentrated on lesions present in more than 10% of cases (Figure 2). There were a total of 117 regions containing 3041 transcripts (Table S1), which is a considerable reduction in complexity from the 30 000 known transcripts. However, it still remains a challenge to identify pathogenetically relevant genes from within this list. The approach of using patterns of expression of these genes is one way of reducing the complexity further, but it has to be realized that potentially important genes may be lost by this approach. We have extended this approach by identifying genes whose patterns of expression change during the transition of normal through MGUS to myeloma plasma cells, which map to the regions of LOH. Using these criteria we have identified 47 genes differentially expressed during this transition. This has defined a more limited number of genes which may be potentially relevant. We have paid more attention to potential tumor suppressor genes, mapping to regions of LOH, with decreased levels of expression in the LOH cases. However, using this approach only one known tumor suppressor gene has been identified, *GABARAP*, which has reduced expression in breast cancer cell lines.[68] This gene is located on 17p, a region only rarely deleted in this series of presentation myeloma samples. Additional, as yet uncharacterized, genes from this list are targets for further investigation.

SNP array analyses can successfully be performed on myeloma plasma cells and are able to accurately define regions of LOH with and without copy number change. We have been able to validate this technology by identifying regions of copy number change previously described in myeloma and in comparison with FISH results from our cases. Using this technology we have been able to show that UPD is an important mechanism in myeloma pathogenesis that can contribute to the inactivation of tumor suppressor genes. This suggests that DNA double-strand breaks and their repair at loci other than the immunoglobulin genes are important in the progression of myeloma. The importance of this mechanism varies between the distinct subtypes of myeloma. In particular, the t(11;14) subgroup seems to be distinctly different in this respect, having fewer regions of LOH and UPD events, a feature which possibly explains the difference in outcome of this subgroup.

A major aim in cancer biology is to understand the full spectrum of gene changes giving rise to tumor initiation and progression. Despite the completion of the human genome project, addressing this issue remains a significant problem and the complexity of available data needs to be reduced to give us a chance of interpreting it. Uniquely in this study we have been able to integrate genotype and copy number data with global gene expression data from the same primary myeloma patient samples to generate lists of genes not only dysregulated in myeloma generally but which are differentially expressed and located within the chromosomal regions of interest. We have documented these genes and their chromosomal location in Tables S1 and S2, providing a focus for further studies to identify and fully characterize genes from within these regions as key oncogenes or tumor suppressor genes in myeloma.

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