Evaluation of 13q14 Status in Multiple Myeloma by Digital Single Nucleotide Polymorphism Technology

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Chromosome 13q deletions are common in multiple myeloma and other cancers, demonstrating the importance of this region in tumorigenesis. We used a novel single nucleotide polymorphism (SNP)-based technique, digital SNP (dSNP), to identify loss of heterozygosity (LOH) at chromosome 13q in paraffin-embedded bone marrow biopsies from 22 patients with multiple myeloma. We analyzed heterozygous SNPs at 13q for the presence of allelic imbalances and examined the results by sequential probability ratio analysis. Where possible, dSNP results were confirmed by fluorescence *in situ* hybridization. Using dSNP, we identified 13q LOH in 16/18 (89%) (95% Confidence Interval; 65%, 99%) patients without the need for neoplastic cell enrichment. In 8/16 (50%) cases, either partial or interstitial patterns of LOH were observed. Both fluorescence *in situ* hybridization and dSNP data proved concordant in just 3/9 cases. Five of the six discrepancies showed LOH by dSNP occurring beyond the boundaries of the fluorescence *in situ* hybridization probes. Our findings show that dSNP represents a useful technique for the analysis of LOH in archival tissue with minimal infiltration of neoplastic cells. The high-resolution screening afforded by the dSNP technology allowed for the identification of complex chromosomal rearrangements, resulting in either partial or interstitial LOH. Digital SNP represents an attractive approach for the investigation of tumors not suitable for genomic-array analysis. (J Mol Diagn 2009, 11:450–457; DOI: 10.2353/jmoldx.2009.090027)

Multiple myeloma (MM) is a post germinal center B-cell malignancy characterized by the accumulation of plasma cells in the bone marrow, chromosomal instability, and chromosomal translocations involving the immunoglobulin heavy chain locus. This disorder is estimated to account for 10% of all hematological malignancies¹ and has an extremely variable prognosis, with survival ranging from a few months to more than 10 years.² Despite recent advances in

gene expression profiling, the molecular mechanisms underlying the development of MM remain unclear.

Cytogenetic instability is a key feature of MM, and chromosomal abnormalities are detectable in the majority of cases.³ Several recurrent abnormalities have emerged that appear to influence disease development and progression. These include gains of chromosome regions 1q, 9q, and 11q, losses of chromosome regions 6q, Xp, and Xq,⁴ and translocations involving 14q32.⁵ Deletion of chromosome 13q is one of the most frequent cytogenetic abnormalities associated with MM, occurring in 40% to 50% of cases.^{4,6,7} The majority of deletions involve entire chromosomes or chromosome arms,^{8,9} although partial and interstitial deletions have also been described.^{10,11} Where interstitial deletions are present, they most commonly involve chromosome band 13q14 to 13q 21.^{10,12}

The presence of chromosome 13q deletions has been suggested to be an adverse prognostic factor in MM.^{6,10} This has lead several groups to hypothesize that the 13q14 region may harbor one or more as yet uncharacterized tumor suppressor genes.^{6,10} This region of chromosome 13 has not only been associated with tumorigenesis in MM⁶ but also in chronic lymphocytic leukemia,¹³ myelofibrosis,¹⁴ melanoma,¹⁵ prostate cancer,¹⁶ laryngeal cancer,¹⁷ colon cancer¹⁸ and non-squamous cell lung cancer.¹⁹

Several methods for the detection and characterization of chromosome 13q deletions have been described. These include metaphase cytogenetics,¹⁰ interphase fluorescence *in situ* hybridization (FISH),²⁰ comparative genome hybridization (CGH)⁴ and array-based CGH.²¹ These techniques, although useful, have several limitations. Metaphase cytogenetics requires the presence of a dividing cell population. Since terminally differentiated plasma cells have a low proliferative capacity, conventional cytogenetics

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is only informative in approximately one third of cases.²² FISH and CGH have a higher detection rate,^{4,20} but may not identify cases where the affected area of chromosome is very small. Array-CGH is a highly sensitive technique that can detect very small regions of deletion, however, it is acknowledged to have limited sensitivity for the detection of aberrations present in a low percentage of cells.^{23,24} Since MM samples typically consist of a heterogeneous population of normal and malignant cells, the identified techniques may not be suitable in cases where the myeloma load is low. In MM, therefore, the minimally deleted region on 13q has hitherto remained poorly defined.

We report here the novel application of digital single nucleotide polymorphism (dSNP) analysis²⁵ for the characterization of 13q LOH status in a panel of 22 MM cases. We have previously shown that dSNP compares well with established molecular techniques, including FISH and multiplex ligation-dependent probe amplification, for the

detection of the chromosome aberrations resulting in LOH (In press: JMD08-0167). Digital SNP allows detection of LOH through the direct counting of alleles. The technique relies on the identification of heterozygous single nucleotide polymorphisms (SNPs) in patient samples and the subsequent separation of the heterozygous alleles through the serial dilution of patient DNA. PCR amplification of the heterozygous alleles is performed and the allelic frequencies counted. Deviation from the expected 50:50 ratio for heterozygous alleles represents LOH at that specific locus and is highly suggestive of a deletion. Sequential probability ratio testing²⁶ confirms the significance of such deviations. Digital SNP technology is applicable to both fresh and archival material, and can be used on samples regardless of proliferative state.

The aim of this study was to employ a panel of SNP probes to characterize 13q14 LOH status in archival tissue from a cohort of 22 patients with MM.

Table 1. Sequences of Probes and Primers Used for Digital SNP Analysis

Name	Sequence	Labels
RB-1 F	5'-GTTGCTGGACAGCCTATGGAT-3'	NONE
RB-1 R	5'-AAGGAATTATACCAAAGCAGCTAAGTAA-3'	NONE
RB-1 P(1)	5'-TCAGGTGACTATCTTTTGT-3'	5'-VIC,3'-MGB
RB-1 P(2)	5'-CAGGTGACTATGTTTGT-3'	5'-6-FAM,3'-MGB
ARLTS1 F	5'-AGCCCCCTCCCAAGTGA-3'	NONE
ARLTS1 R	5'-CAGGCTGAGTCTAGAGAGATTCCA-3'	NONE
ARLTS1 P(1)	5'-TCCCAGCAGTGGTC-3'	5'-VIC,3'-MGB
ARLTS1 P(2)	5'-CCCAGCGGTGGTC-3'	5'-6-FAM,3'-MGB
EBPL F	5'-CCCACCCTTCTACTTTTCTGGATTT-3'	NONE
EBPL R	5'-CACCAGCCGGCTTAAAGC-3'	NONE
EBPL P(1)	5'-AAATGGAAGATTGTCTAGAGTA-3'	5'-VIC,3'-MGB
EBPL P(2)	5'-ATGGAAGATTGTCTGGAGTA-3'	5'-6-FAM,3'-MGB
C13Orf1 F	5'-CTCATTAGATCCAATCCCAGTCTT-3'	NONE
C13Orf1 R	5'-CCACTGTAAATGACTAAATTTGAAGTCTT-3'	NONE
C13Orf1 P(1)	5'-AAACTCCCAACAAAG-3'	5'-VIC,3'-MGB
C13Orf1 P(2)	5'-AACTCCCAACAAAG-3'	5'-6-FAM,3'-MGB
KCNRG F	5'-GATCTCTCAAAAATTGATGAGTGTGGTT-3'	NONE
KCNRG R	5'-TCGAGACCTTGCTGTGATTCTG-3'	NONE
KCNRG P(1)	5'-AAAGTCATTTGAATTCC-3'	5'-VIC,3'-MGB
KCNRG P(2)	5'-AAGTCATTTCAATTCC-3'	5'-6-FAM,3'-MGB
DLEU2 F	5'-ACTTCCCTACCTATTCTCTGGAAAA-3'	NONE
DLEU2 R	5'-GGAGCTAGAGGCATTTTGTAAAGCAA-3'	NONE
DLEU2 P(1)	5'-AATCAAGAAATGACACTTT-3'	5'-VIC,3'-MGB
DLEU2 P(2)	5'-CAATCAAGAAATGACACTTT-3'	5'-6-FAM,3'-MGB
DLEU1 F	5'-GCCAGTGTCTAAACTCCAAACAAC-3'	NONE
DLEU1 R	5'-GGCGGTTTAAATGCACGTGTATC-3'	NONE
DLEU1 P(1)	5'-CAATGAGACCTAGTATATG-3'	5'-VIC,3'-MGB
DLEU1 P(2)	5'-CAATGAGACCTGTATATG-3'	5'-6-FAM,3'-MGB
FAM10A4 F	5'-CTACCAGCAGGTGTCATTATGGA-3'	NONE
FAM10A4 R	5'-TTTCTTTCTCACAGCTAGTTCTCTACAAC-3'	NONE
FAM10A4 P(1)	5'-TCTGGAATTTACAGTATTTT-3'	5'-VIC,3'-MGB
FAM10A4 P(2)	5'-CTGGAATTTACACTATTTT-3'	5'-6-FAM,3'-MGB
DLEU7 F	5'-GGGTCTTGAAAGAAAAGTACAGAGCTA-3'	NONE
DLEU7 R	5'-CCACTCAGTTTCCCACACTAA-3'	NONE
DLEU7 P(1)	5'-AAAGAAAGGCATCCCCCAG-3'	5'-VIC,3'-MGB
DLEU7 P(2)	5'-AAAGGCATCGCCCCAG-3'	5'-6-FAM,3'-MGB
SLC15A1 F	5'-GCTGCTGATTTTCAGTGGAGACA-3'	NONE
SLC15A1 R	5'-TTTTGGATTAATCACTCCAGCTT-3'	NONE
SLC15A1 P(1)	5'-TCTAGATGCAAGTATCTG-3'	5'-VIC,3'-MGB
SLC15A1 P(2)	5'-TAGATGCAAAATATCTG-3'	5'-6-FAM,3'-MGB
UBAC2 F	5'-GAATCCTAAGATGCTTAATTTTGTAAGTTTGCA-3'	NONE
UBAC2 R	5'-GGTGCATGATGACAGTGAAAA-3'	NONE
UBAC2 P(1)	5'-CTCGGATCATATTTAG-3'	5'-VIC,3'-MGB
UBAC2 P(2)	5'-CTCGGATCGTATTTAG-3'	5'-6-FAM,3'-MGB

Probes are labeled 5' with 6-FAM or VIC and 3' with a minor groove binding protein (MGB) to enhance probe specificity. Nucleotides representing the SNPs under investigation are highlighted in bold.

Materials and Methods

Subjects

Twenty-two patients with MM, diagnosed and treated at the Royal Devon and Exeter Hospital were included in this study. The study protocol had received prior approval by the local research ethics committee and all patients provided written informed consent in accordance with the Declaration of Helsinki. The panel of 22 MM anonymized cases (MM01 to MM22) was collected from 2002 to 2007. All patients were over 16 years old and had an established diagnosis of MM with documented bone marrow involvement.

Assessment of Myeloma Load

The myeloma load in each sample was determined by CD138 staining of two 2 μ mol/L sections framing the ten thick sections (10 μ mol/L) used for DNA extraction. Assessment of the myeloma load was performed by a hematopathologist.

DNA Extraction and Quantification

DNA was extracted from 10 paraffin sections of 10 μ mol/L thickness using a modified protocol of the Qiagen QIAamp tissue kit (Qiagen, Crawley, UK).²⁷ DNA sam-

ples were quantified using a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington) and assessed for integrity using the BIOMED 2 specimen control size ladder, which yields products of 84, 96, 200, 300, 400, and 600 bp (Invivoscribe, San Diego, CA).

Assay Design and Validation

A panel of 11 SNP markers that covered the region of 13q12 to 13q34 was chosen on the basis of high population heterozygosity (0.3 to 0.5). Probes and primers for these SNPs were designed by the Applied Biosystems Assays-on-Demand service (Applied Biosystems, Foster City, CA) (Table 1). In each case, allele specific probes were labeled 5' with either 6-FAM or VIC and 3' with a minor groove binding protein serving as a non-fluorescent quencher. Assays were validated using standard curves generated from seven (1:2) serial dilutions of heterozygous DNA to ensure equivalent amplification and probing efficiency, and by amplification of homozygous individuals of both genotypes to ensure that background fluorescence was within acceptable limits.

dSNP Validation

To determine the validity of the approach, an artificial cell mixture was made using known amounts of homozygous DNA diluted in DNA from an unrelated heterozygous individual. Briefly, DNA samples were diluted to 2.5

Figure 1. Illustration of digital single nucleotide polymorphism (dSNP) analysis. **A:** Schematic of dSNP results for a theoretical sample with no allelic imbalance. Wells scoring as allele 1 are marked in light gray, wells scoring as allele 2 are marked in dark gray. Wells containing both alleles 1 and 2 are hatched while those with no signal are blank. The total number of informative alleles is 36 (18 allele 1 and 18 allele 2). The allele frequency of both alleles is thus 0.50. **B:** Schematic of dSNP results for a theoretical sample showing allelic imbalance. Wells scoring as allele 1 are marked in light gray, wells scoring as allele 2 are marked in dark gray. Wells containing both alleles 1 and 2 are hatched while those with no signal are blank. The total number of informative alleles is 36 (32 allele 1 and 4 allele 2). The major allele is thus allele 1, with an allele frequency of 0.89.

Figure 2. Sequential Probability analysis for detection of loss of heterozygosity (LOH) at 13q. The statistical evidence that an observed skew in allele frequency represents LOH is assessed by plotting the major allele frequency on the y axis against the number of alleles tested on the x axis. Threshold curves are calculated for a given tumor load based on the likelihood ratio of 8. Points plotted above the upper curve represent LOH, while those below the lower curve represent no LOH (see text for further explanation). The different curves represent different ratios of non-neoplastic to neoplastic cells as follows. **(A)** = 50:50 mixture; **(B)** = 60:40 mixture; **(C)** = 70:30 mixture; **(D)** = 80:20 mixture.

mmol/L, and plated out on a 96 well real-time PCR plate. PCR reactions were performed in a total volume of 8 μ l including 4 μ l universal master mix (no AMPerase) (Applied Biosystems), assay mix (0.9 μ mol/L probe, 1.8 μ mol/L each primer) and 2.5pg/ μ l DNA. PCR cycles were 50°C for 2 minutes and 95°C for 10 minutes, followed by 60 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Reactions were performed on the ABI Prism 7000 platform. Following real-time amplification, appropriate baseline and threshold levels were set and samples were designated as allele 1, allele 2, allele 1 + 2 or no product detected.

Initial Assessment of Sample Genotype

All 22 MM samples were amplified by real-time PCR to identify individuals heterozygous for each SNP. PCR was performed in 10 μ l reactions including 5 μ l universal master mix, no AMPerase) (Applied Biosystems), assay mix (0.9 μ mol/L probe, 1.8 μ mol/L each primer) and 40 ng of DNA. PCR cycles were 50°C for 2 minutes and 95°C for 10 minutes, followed by 60 cycles of 95°C for 15 seconds and 60°C for 1 minute. Following amplification, the genotype of each sample was determined for each SNP as outlined above.

Calculation of Optimal DNA Dilution

The optimal DNA concentration to yield good separation of alleles was calculated from the average molecular weight of a mole of base (342 g), the number of molecules in a mole of substance (the Avogadro constant; 6.023×10^{23}) and the number of bases in the human genome (3×10^9). However, since this figure takes no account of the presence of PCR inhibitors or DNA degradation in the sample, optimal dilution factors were further determined empirically.

dSNP Analysis

Optimally diluted DNA samples were plated out in ABI optical plates and amplified on the ABI Prism 7000 plat-

form. PCR reactions were performed in a total volume of 8 μ l including 4 μ l universal master mix (no AMPerase) (Applied Biosystems), assay mix (0.9 μ mol/L probe, 1.8 μ mol/L each primer) and 2 μ l appropriately diluted DNA. PCR cycles were 50°C for 2 minutes and 95°C for 10 minutes, followed by 60 cycles of 95°C for 15 seconds and 60°C for 1 minute. Following amplification, each well was genotyped as outlined above.

Sequential Probability Analysis

Likelihood analysis was used to assess the strength of evidence for deviation from a 50:50 distribution of alleles. The specific method used was the sequential probability ratio testing, as previously described.²⁵ This technique allows two probabilistic hypotheses to be compared as data accumulates. A sample with no LOH is expected to show allelic balance, with the two heterozygous alleles appearing at approximately the same frequency (null hypothesis H^0) (Figure 1A). A sample with LOH will have allelic imbalance (alternative hypothesis H^1) with over-representation of one allele (Figure 1B). The degree of imbalance will be directly proportional to the number of clonal plasma cells that are present in the sample and that harbor the aberration. Threshold curves representing the two hypotheses are generated for a specific tumor load based on a predetermined likelihood ratio. Analysis is concluded by plotting the number of informative alleles tested against the major allele ratio (Figure 2). If the point is plotted above the upper threshold curve, it is considered as evidence of allelic imbalance and hence LOH at that locus. Points plotted below the lower threshold curve are categorized as having allelic balance and therefore no LOH. Points falling between the threshold curves were analyzed as previously described.²⁸ Briefly, dSNP experiments were performed until the number of informative alleles exceeded 150. The mid-point value between the two hypotheses was then calculated for the given tumor load. When the major allele ratio exceeded the mid-point value it was considered as evidence of LOH, while ratios falling below the mid-point value were assigned to the no LOH category.

Figure 3. Schematic representation of partial and interstitial loss of heterozygosity (LOH) observed at 13q. The sample number is given at the left side of the figure and the markers tested are shown at the top. Markers colored in black represent loci with LOH determined by dSNP analysis, while those in white represent loci with no LOH. **A:** Patients with partial LOH at 13q. **B:** Patients with interstitial patterns of LOH/heterozygosity at 13q. In these cases, large regions of LOH were disrupted by small, interstitial patterns of heterozygosity.

Table 2. Identification of LOH at Chromosome 13q by dSNP Analysis of MM Samples

Continued

Myeloma load	13q14								
	RB1	ARLTS1	EBPL	C13orf1	KCNRG	DLEU2	DLEU1	FAM10A4	DLEU7
MM01 >50%	0.53 (0.62; <u>121</u>)	—	—	—	0.79 (0.66; <u>57</u>)	—	—	—	0.75 (0.64; <u>75</u>)
MM02 >50%	—	—	0.73 (0.63; <u>101</u>)	—	—	—	0.55 (0.62; <u>130</u>)	—	0.86 (0.72; <u>29</u>)
MM04 >30%	—	—	—	—	—	0.92 (0.86; <u>25</u>)	—	—	—
MM06 >90%	—	—	—	—	—	0.51 (0.66; <u>57</u>)	—	0.51 (0.66; <u>51</u>)	—
MM07 >30%	—	—	0.86 (0.70; <u>49</u>)	—	—	—	—	—	—
MM09 >90%	—	—	—	—	—	—	—	0.81 (0.68; <u>43</u>)	0.81 (0.71; <u>32</u>)
MM10 >30%	—	—	—	0.74 (0.71; <u>47</u>)	0.50 (0.58; <u>205</u>)	—	—	—	0.84 (0.84; <u>26</u>)
MM11 >80%	0.95 (0.65; <u>60</u>)	0.67 (0.66; <u>57</u>)	—	—	—	—	—	—	—
MM12 >80%	0.89 (0.73; <u>27</u>)	—	—	—	—	—	0.71 (0.69; <u>38</u>)	—	—
MM13 >20%	0.57 (0.53; <u>211</u>)	—	0.58 (0.53; <u>151</u>)	—	—	—	—	—	—
MM14 >20%	—	—	—	—	—	—	0.55 (0.53; <u>220</u>)	—	—
MM15 >20%	—	—	—	—	—	—	0.55 (0.53; <u>241</u>)	—	—
MM16 >90%	—	—	—	0.58 (0.60; <u>257</u>)	0.51 (0.63; <u>85</u>)	0.55 (0.60; <u>219</u>)	—	—	—
MM17 >80%	0.54 (0.61; <u>150</u>)	—	—	—	0.52 (0.61; <u>149</u>)	0.55 (0.61; <u>151</u>)	—	—	0.55 (0.61; <u>173</u>)
MM18 >70%	—	—	0.65 (0.63; <u>91</u>)	—	—	—	0.51 (0.61; <u>160</u>)	—	—
MM20 >40%	0.54 (0.59; <u>219</u>)	—	0.54 (0.59; <u>232</u>)	0.50 (0.60; <u>167</u>)	—	0.61 (0.60; <u>172</u>)	—	—	—
MM21 >40%	—	—	0.61 (0.61; <u>121</u>)	—	—	—	—	—	0.53 (0.60; <u>159</u>)
MM22 >50%	—	—	0.61 (0.61; <u>166</u>)	—	—	—	0.53 (0.62; <u>129</u>)	—	0.62 (0.62; <u>124</u>)

The multiple myeloma load and major allele frequencies are given for informative markers for samples MM01 to MM22. Threshold limits, above which results are consistent with LOH are given in italics, and the number of informative alleles in each assay underlined. Samples showing evidence of LOH are marked in bold.

A likelihood ratio of >8 was accepted as evidence of LOH since it provides a posttest probability of LOH of 89%, if the pre-test probability of the presence of LOH at 13q is assumed to be 50%. Since sequential probability ratio testing allows for repeat testing, the posttest probability of LOH can be increased further by testing each sample with several allelic probes.

The proportions P of the major allele for a likelihood ratio of 8 for the presence of LOH in test samples with varying myeloma cell infiltrations were calculated according to the formula $P = \log(16)/(n \cdot \log[(100-m)/100]) + \log([100-m]/[100-m/2])/\log([100-m]/100)$, where n = total number of alleles counted and m = the percentage of myeloma infiltration in the biopsy. The results of the equation were tabulated with Microsoft Excel 2000 software.

Confirmation of Results with FISH

In nine cases, suitable samples were available for FISH analysis of chromosome 13q. FISH analysis was performed by Wessex Regional Cytogenetics Laboratory, according to the methods outlined by Ross et al.²⁹ The method employs two probes (RB1 and D13S319) mapping at chromosome band 13q14.

Results

DNA Extraction and Quantification

DNA was extracted from paraffin sections as described above. The DNA samples were shown to be suitable for PCR by the amplification of the 400 ± 600 bp products in the BIOMED 2 specimen control size ladder.

Assay Design and Validation

Real-time PCR assays were designed and validated for 11 SNPs spanning the 13q14.2 to 13q34 region. The median distance between the SNP probes at 13q14 was

90 kb (Range; 10 kb to 1287 kb). All assays were deemed suitable for further analysis on the basis of yielding reliable and specific amplification with an efficiency difference of less than 0.5 between alleles. All assays demonstrated a linear relationship between crossing point and DNA concentration over seven 1:2 serial dilutions (data not shown).

dSNP Validation

Following PCR amplification of a mixed sample of 30% 'LOH' cells (2/2) and 70% 'no-LOH' cells (1/2), a total of 90/174 (52%) alleles were informative for dSNP analysis. The frequencies of allele 1 and allele 2 were found to be 0.33 (30/90 informative wells) and 0.67 (60/90) respectively. Using sequential probability ratio testing analysis on a test sample of 90, the lower limit for acceptance of LOH with a likelihood ratio of 8 is 0.63. The observed major allele ratio of 0.67 was therefore consistent with the presence of LOH at 13q in this sample.

Assessment of SNP Genotype for Each Sample

All samples were found to be heterozygous for at least one of the 11 SNPs within the panel and were therefore suitable for dSNP analysis. The median number of heterozygous SNPs per patient was 4 (Range; 2, 7). In patients with more than one informative SNP at 13q14, the median distance between SNPs per patient was 695kb (Range; 52 kb to 1738 kb).

Calculation of Optimal DNA Concentration for Study

The average mass of one molecule of DNA was calculated as 2.6×10^{-12} g. Therefore, a DNA concentration of 2.6×10^{-12} pg/microlitre should yield one template molecule per 1 µl, assuming that no DNA degradation has taken

Table 2. *Continued*

13q32	
SLC15A	UBAC2
—	—
0.65 (0.62; 118)	—
—	—
0.67 (0.61; 115)	—
—	—
—	—
0.58 (0.53; 165)	—
0.80 (0.71; 70)	—
0.68 (0.63; 124)	—
—	0.64 (0.63; 81)
—	—
0.65 (0.61; 115)	0.59 (0.58; 191)
—	—
0.67 (0.65; 67)	—

place. In practice, the presence of PCR inhibitors left from paraffin extraction and the occurrence of variable amounts of DNA degradation in the samples meant that the optimal DNA concentrations ranged from 2 pg/ μ l to 50 pg/ μ l.

Assessment of Myeloma Load

In the majority of cases (18/22), the extent of bone marrow infiltration by myeloma cells ranged from >20% to >90% (Table 2). In four instances (MM03, MM05, MM08, and MM19) the myeloma load was $\leq$ 10%. These cases were excluded from dSNP analysis on the basis that too many informative alleles would be required to assess the strength of evidence for deviation from 50:50 distribution of alleles by likelihood analysis.

dSNP Analysis

The LOH status of selected informative markers spanning the region 13q14.2 to 13q34 was determined for each of the MM patients with >20% myeloma load by dSNP analysis. The calculated major allele frequencies, number of alleles analyzed and threshold allele frequencies are given in Table 2. A total of 16/18 (89%) (95% CI; 65%–99%) samples showed statistically significant deviations from a balanced allele frequency indicating the presence of LOH (Figure 2, A–D). The extent of LOH

varied considerably between patients and no common region of LOH was evident. In four cases (MM04, MM09, MM11, and MM12) we were unable to define the extent of LOH further due to a lack of informative markers. In four cases (MM07, MM13, MM14, and MM15) LOH analysis was suggestive of chromosome 13 monosomy. The remaining 8/16 (50%) cases showed evidence for partial or interstitial LOH (Figure 3, A and B). The dSNP approach enabled successful identification of LOH in patients with myeloma loads as low as 20% (MM13, MM14, and MM15).

Assessment of 13q Deletion Status by FISH

Of the nine patients for whom samples were available for FISH testing, three (MM06, MM09, and MM14) showed complete concordance with the dSNP data. Six discrepancies (MM01, MM07, MM10, MM12, MM18, and MM21) were observed between the dSNP and FISH techniques. Each discrepant case showed evidence of LOH by dSNP analysis that was not apparent by FISH. In 5/6 discrepant cases, the identified LOH occurred beyond the boundaries of the FISH probes (Figure 4). In four of these cases (MM01, MM10, MM18, and MM21) heterozygous SNPs at the specific loci targeted by the FISH probes did not show LOH by dSNP analysis.

Discussion

We demonstrate here the first application of digital SNP technology to the analysis of MM samples with heterogeneous cell populations without prior microdissection. We have previously shown that dSNP represents a valid approach for the identification and characterization of chromosome 13q LOH, comparing well with established molecular techniques, including FISH and multiplex ligation-dependent probe amplification.³⁰

Through the application of dSNP technology, we identified 13q LOH in 16/18 (89%) (95% CI; 65%–99%) patients with MM. Loss of heterozygosity was identified in samples with as little as 20% myeloma cell infiltration, without the need for any pretreatment to reduce cellular heterogeneity. In contrast to techniques such as FISH, the analysis of heterogeneous cell populations by dSNP cannot definitively confirm the cellular origin of the identified chromosomal abnormalities. This represents a potential limitation of the dSNP technique.

Figure 4. Schematic representation of cases with discrepant dSNP and FISH results. The sample number is given at the left side of the figure and the markers tested are shown at the top. Markers colored in black represent loci with LOH determined by dSNP analysis, while those in white represent loci with no LOH. The boxes denoted RB1 and D13S319 illustrate the loci of the FISH probes. Each case showed evidence of LOH by dSNP occurring beyond the boundaries of the FISH probes. In four cases (MM01, MM10, MM18, and MM21) heterozygous SNPs at the specific loci targeted by the FISH probes did not show LOH by dSNP analysis, consistent with the FISH result.

The high incidence of 13q LOH detected in our cohort contrasts with most data in the current literature. The majority of studies report 13q deletions in around 40% to 50% of MM cases.^{4,6,7} Our findings may indicate that 13q aberrations are more common than currently believed and that a focused, high-resolution approach is necessary for their accurate detection. For example, a previous study using high-resolution analysis of the 13q region identified a comparable incidence of 13q abnormalities. Shaughnessy et al³¹ reported chromosome 13 deletions in 86% of myeloma patients when applying a panel of 11 FISH probes (five of which targeted chromosome band 13q14) along the long arm of chromosome 13. Alternatively, the discrepancy observed may reflect a sample bias introduced by the small sample size included in this study. Our small patient cohort may not be entirely representative of the MM population and, as such, inclusion of additional cases may have resulted in an incidence of 13q deletions that more accurately reflects current data.

The high-resolution screening achieved by the dSNP approach was accomplished through the application of a panel of 11 SNP probes targeting the 13q14.2 to 13q34 region. The median distance between the probes targeting 13q14 was just 90kb (Range; 10 kb to 1287 kb). This permitted LOH analysis to extend beyond the boundaries of the FISH probes that are used diagnostically, thereby giving additional information about the 13q14 region. As such, 8/16 (50%) patients showed evidence of partial or interstitial LOH (Figure 3, A and B). Most previous studies using FISH for the analysis of 13q14 report that chromosome 13 deletions are consistent with monosomy 13 in the vast majority of cases.^{8,9} However, an alternative approach using genotypic analysis of polymorphic markers along chromosome 13 reported that interstitial 13q deletions may be as common as monosomies,¹¹ thus supporting our findings. Despite the high incidence of partial and interstitial LOH within our patient cohort, dSNP analysis did not identify a common region of LOH.

An attractive alternative approach for the high-resolution analysis of the MM genome is array-CGH. The application of this technique has successfully demonstrated chromosome copy number imbalances in all primary MM cases.²¹ However, this study reports chromosome 13q losses in just 38% of cases, contrasting with the findings of our study. This discrepancy may be attributed to the relatively small sample numbers of both studies. Despite the proven utility of array-CGH, the technique is acknowledged to have limited sensitivity for the detection of chromosomal aberrations present in a low percentage of cells.²⁴ It may therefore not be suitable for the analysis of archival tissues with low numbers of neoplastic cells.

A comparison of the dSNP and FISH techniques was performed on a subset of nine patients. Initially, discrepancies between techniques were observed for 6/9 patients. In each discrepant case dSNP analysis identified 13q LOH that was not apparent by FISH. Close inspection of the discrepancies showed that in 5/6 cases dSNP analysis identified LOH at loci occurring beyond the boundaries of the FISH probes and could therefore not be assessed accurately by the FISH technique. In four of these cases (MM01, MM10, MM18, and MM21) informa-

tive SNPs occurring at the specific loci targeted by the FISH probes did not show LOH by dSNP analysis, consistent with the FISH results (Figure 4).

Interestingly, each of the four cases showing interstitial LOH had a heterozygous region between the *KCNRG* and *DLEU1* genes. Identification of such rearrangements strengthens the evidence for the existence of recombination hot spots within this region of 13q14. Previous work in chronic lymphocytic leukemia has identified prominent clustering of breakpoints on both sides of the *MIR15A* and *MIR16-1* genes, which are situated at this locus,³² supporting this hypothesis.

We have shown that dSNP technology represents a useful approach for the detection and characterization of LOH in archival tissue samples containing a variable proportion of neoplastic and normal cells. The sensitive, high-resolution screening afforded by this technique allows identification of complex chromosomal rearrangements resulting in partial or interstitial LOH. Digital SNP represents an attractive approach for the investigation of tumors not suitable for genomic-array analysis.

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