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BRIEF COMMUNICATION

Delineation of the Minimal Region of Loss at 13q14 in Multiple Myeloma

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Previous studies have focused on the incidence and prognostic implications of 13q14 deletions in multiple myeloma (MM), but none has sought to delineate the minimal common deleted region (CDR). In an effort to do so, dual-color interphase fluorescence in situ hybridization (FISH) was applied on 82 myeloma cases, initially by use of three probes for 13q14 (*RB1, D13S319,* and *D13S25*). Deletions were detected in 29/82 (35.4%) cases, and all except one were monoallelic. Subsequently, contiguous YACs, PACs, and a BAC spanning the 13q14–q21 region were employed for deletion mapping in addition to a 13q telomere probe. Large deletions extending to the 13q34 region were found in 55% of the deleted cases, whereas an additional 13.8% showed loss of both 13q34 and 13q14 regions with retention of 13q21. A CDR of approximately 350 kb was identified at 13q14 with the proximal border approximately 120 kb centromeric from *D13S319,* encompassing an area rich in expressed sequence tagged sites and containing *DLEU1, DLEU2,* and *RFP2* genes. Direct sequencing of the *RFP2* gene revealed no mutations in six patients and four MM cell lines harboring deletions of the CDR. However, a role for *RFP2* in the pathogenesis of MM cannot yet be excluded, given that alternative mechanisms such as haploinsufficiency remain possible.

Multiple myeloma (MM) is a plasma cell malignancy originating from B-lineage clonogenic cells and accounting for 10% of all hematologic malignancies. Despite the observation that virtually all MM patients are chromosomally abnormal, the molecular basis of this disease remains elusive (Flactif et al., 1995; Zandecki et al., 1996; Kastrinakis et al., 2000). The most commonly detected clonal chromosomal abnormalities consist of rearrangements of 1q and 14q32 and partial or total loss of chromosome 13 (Cigudosa et al., 1994; Sawyer et al., 1995; Bergsagel et al., 1996; Calasanz et al., 1997).

Previous studies have suggested that 13q deletions are an early event in the development of monoclonal gammopathies and point to their involvement in the evolution of monoclonal gammopathy of undefined significance into overt MM (Avet-Loiseau et al., 1999; Konigsberg et al., 2000a). Cytogenetic and fluorescence in situ hybridization (FISH) studies have detected the presence of chromosome 13 deletions in 20–86% of MM cases, more commonly within the 13q14 region (Tricot et al., 1995; Chang et al., 1999; Desikan et al., 2000; Shaughnessy et al., 2000). An adverse prognostic role linked to these deletions has been reported by several groups, supporting the suggestions of the presence of an MM tumor-suppressor gene(s) (TSG) at 13q14 (Tricot et al., 1995; Desikan et al., 2000; Zojer et al., 2000; Facon et al., 2001). There has been no attempt, however, accurately to delineate the borders of the minimal common deleted region in MM, which is the essential step toward identifying the culprit gene residing in this region.

Hence, in the present study, we endeavored to identify the common region of deletion (CDR) in MM by use of a panel of contiguous probes spanning the 13q14–21 region. The identified region of interest was found to consist of the *RFP2* gene; thus, we performed mutational analysis by direct sequencing of the open reading frame of this gene in patient samples and cell lines with deletions of the CDR. The purpose of this was to examine the possibility that *RFP2* would be the TSG implicated in the pathogenesis of myeloma through the classical "two-hit" model.

Bone marrow samples were obtained from 82 consecutive patients attending the myeloma clinic at the Royal Marsden Hospital, Sutton, UK, be-

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TABLE 1. Frequency of Deletions in the Preliminary Experiments, by Use of the Three Locus-Specific Probes

No. of cases	*RB1*	*D13S319*	*D13S25*
20	deleted	deleted	deleted
4	deleted	deleted	N
2	N	deleted	deleted
3	N	deleted	N

N, not deleted.

tween 1997 and 2001. Thirty-eight patients were newly diagnosed and a further eight patients had not received prior therapy. The median age was 58 years (range: 29–83); 27 were female and 55 were male. The range of plasma cells in the samples was 10–95% (mean = 40%).

Results of conventional karyotyping were available for 53 patients. Chromosome arm 13q abnormalities were found in 9/15 with clonal abnormalities. Dual-color FISH was carried out on separated mononuclear cells as described previously (Gruszka-Westwood et al., 2001), in all 82 patients. Initially, three locus-specific probes (LSI), *RB1*, *D13S319*, and *D13S25* (Vysis, Richmond, UK), were used as anchoring points in the 13q14 region. Cutoff levels for all LSI probes ranged between 7.7 and 9% based on scoring of peripheral blood and bone marrow controls. A reference probe hybridizing to chromosome arm 21q was used to control for hybridization efficiency. Twenty-nine patients (35.4%) were found to harbor deletions with at least one of these 13q14 probes. The percentage of cells showing deletions ranged between 12 and 96% for the three probes. All deletions were monoallelic except in one case (mm55) showing biallelic deletion of *RB1* in a proportion of cells with 13q deletions. Twenty of 29 (69%) cases had deletions with all three probes, and the most frequently involved locus was *D13S319*, which was deleted in all cases, followed by *RB1* (24/29 = 83%) and *D13S25* (22/29 = 76%) (Table 1). All cases with karyotypic abnormalities of 13q were confirmed by FISH, except one that had a karyotype of 73–76,XXX with complex abnormalities including add(13)(q3)×2 and no normal copies of chromosome 13. Two signals were detected by FISH analysis, and so there was no evidence of a deletion.

Subsequent to results obtained from the initial FISH experiments, the following probes were applied to delineate the proximal and distal borders of the CDR: yeast artificial chromosomes (YACs) 851f1, 908f4, and 954c12 (Bezieau et al., 1998); P1 artificial chromosomes (PACs) 157m7, 246m14, and 55-o6 (Kalachikov et al., 1997), obtained from the Human Genome Mapping Project; and bacterial artificial chromosome (BAC) 34f20, which maps to the location of *DLEU1*, *DLEU2*, and *RFP2*, kindly provided by Dr. Martin Corcoran (Royal Bournemouth Hospital, Bournemouth, UK). These probes spanned the 13q14–q21 region in a contiguous manner with some overlap between them. In addition, a telomere 13q (LSI) probe (*D13S327*; Vysis) was used to identify the cases with major deletions. Figure 1A shows the size and approximate location of each probe. DNA extraction was performed for the YACs, PACs, and BAC, followed by nick translation according to standard methods.

Probes were labeled with either biotin-16-dUTP or digoxigenin-11-dUTP (all reagents from Roche, UK) and used in dual-color experiments (Fig. 2). The cutoff levels for defining deletions in these probes ranged between 5.6 and 8.4%, based on the same controls as above. Percentages of deleted cells with these probes ranged between 14 and 89%. On the basis of the pattern of deletions obtained, a common deleted region of approximately 350 kb, which includes BAC 34f20 and PAC 246m14, was determined (Table 2 and Fig. 1B). This region was defined by use of the informative cases that harbored smaller deletions within the 13q14–q21 region. The centromeric border was delineated by cases mm12 and mm43, and the telomeric border by cases mm12, mm52, mm6, mm50, and mm66. Sixteen cases (55%) had major deletions extending down to 13q34, evidenced by loss of both y954c12 and the telomeric 13q probe. Lack of hybridization with these two probes may denote either 13q- or monosomy 13. Four other cases (13% of all deletions) with deletions of at least two LSI probes had loss at 13q34, with a preserved 13q21 region, as indicated by retention of the y954c12 signal (Table 2).

Since the CDR included the locus of the *RFP2* gene, mutation analysis was performed by direct sequencing of the coding exon of this gene (exon 3) and flanking introns. This was performed on genomic DNA from six of the MM patients and four myeloma cell lines (Karpas620, U266, LP1, RPMI 8266), who were found to harbor deletions of the CDR, in addition to three myeloma (HS Sultan, IM9, OPM2) and five non-myeloma (Jurkat, JVM2, HL60, MOLT4, CEM) control cell lines (Drexler, 2001).

Primer design was done with the assistance of the Primer 3 program (www.genome.wi.mit.edu/genome_software/other/primer3.html). Sequences of the primers were as follows: primer 1: forward 5′-GCGGATTAAATTTGCTTTGG-3′, reverse

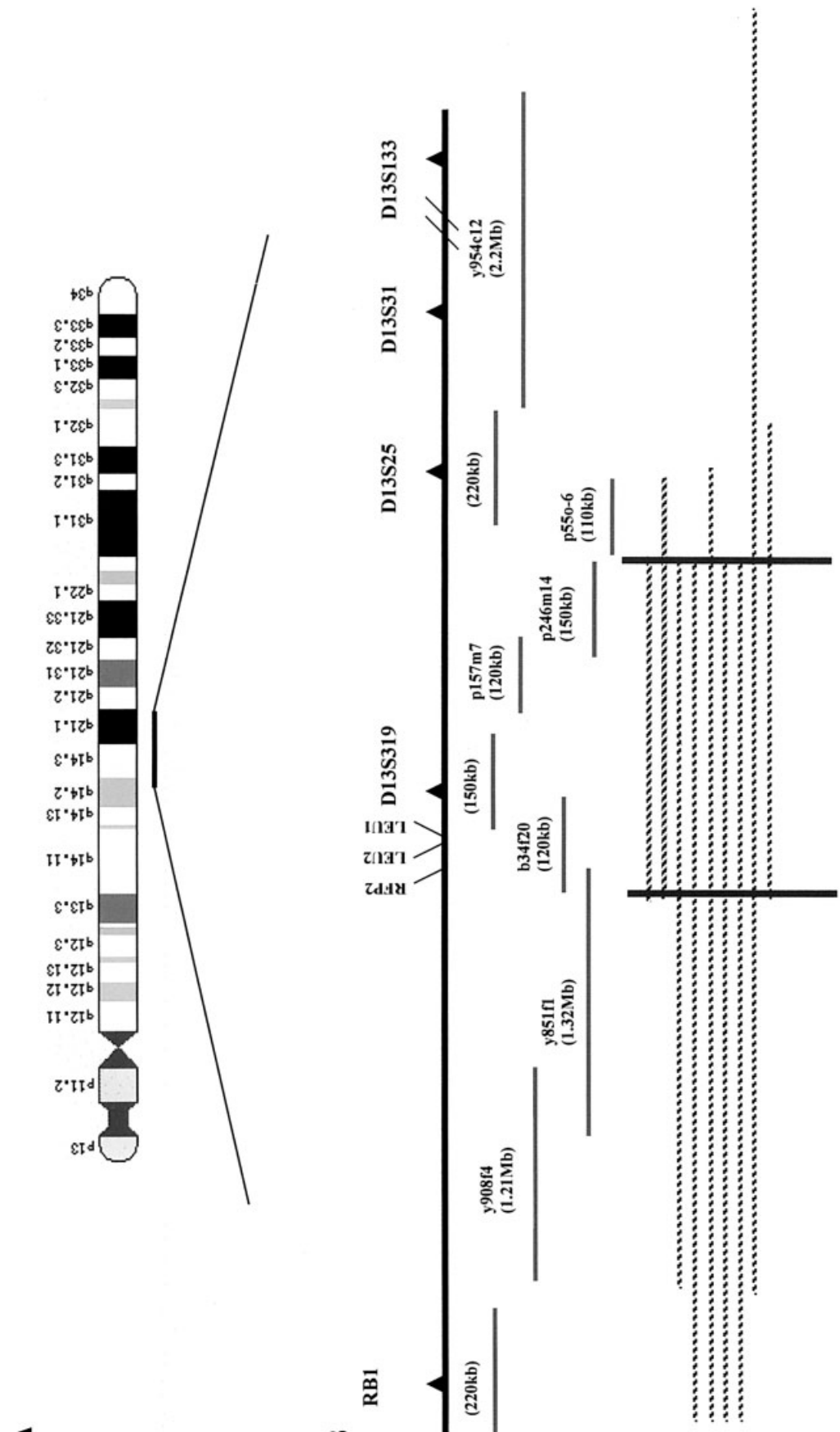

Figure 1. Diagramatic representation of the probes used and the results of deletion mapping at 13q14–13q21. **A:** Probes used at chromosome 13q showing their relative sizes, positions, and overlaps. The three LSI probes at 13q14 (*RB1*, *D13S319*, and *D13S25*) are drawn below their respective loci. **B:** Each patterned line represents the deletion mapped from each informative patient who was found initially to harbor deletions with less than three LSI probes. The size of the commonly deleted region determined from this group of patients is approximately 350 kb, and its boundaries are denoted by the two solid vertical lines.

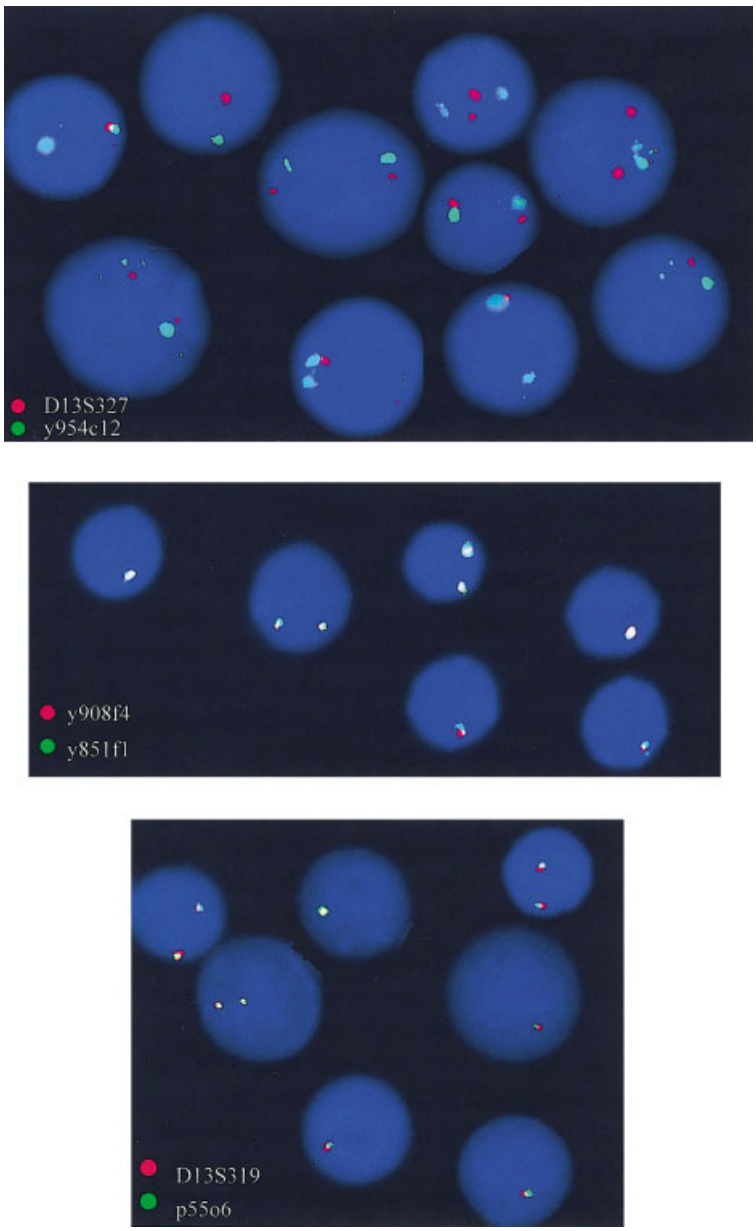

Figure 2. Dual-color FISH experiments using probes for chromosome 13q in MM. The top image shows an example of a heterogeneic population with monoallelic deletion of both y954c12 and telomeric 13q probe in some cells, whereas others show monoallelic deletion of the telomeric 13q probe only with retention of y954c12.

5′-ACAAGGAATTCCGCACACTC-3′, primer 2: forward 5′-ATGTGATGGAGCTGCTTGAA-3′, reverse 5′-GGCAGAAAATGTTGAGAGGC-3′, primer 3: forward 5′-GGCAGCCTCTCAACATTTTC-3′, reverse 5′-TCATGGTCTCAAAGTCAGACAGA-3′, primer 4: forward 5′-TTGAGACCATGAAACTTGCTG-3′, reverse 5′-GCCAGTGTCTTGAGGCAAAG-3′, primer 5: forward 5′-CTTTGCCTCAAGACACTGGC-3′, reverse 5′-GCCACATTGTTCAGTACCACC-3′, primer 6: forward 5′-CTGGGAACAGGTGACAGATG-3′ reverse: 5′-TGACTAGAATCGTTGACCAAATC-3′.

Annealing temperatures were 59°C for all primers, except primer 5, which was 61°C. Otherwise, all PCR and sequencing reaction conditions were as those described elsewhere (Gruszka-Westwood et al., 2001). Despite the high sensitivity of this approach, no mutations were detected in either patients or cell lines.

There have been several reports studying 13q14 in MM by use of interphase FISH; however, none has attempted to delineate the common deleted region (Chang et al., 1999; Avet-Louseau et al., 2000; Desikan et al., 2000; Zojer et al., 2000; Facon et al., 2001; Fonseca et al., 2001). These studies

TABLE 2. Results of Deletion Mapping Experiments by FISH*

Case	PC%	Karyotype	RB1	y908f4	y851f1	b34f20	D13S319	p157m7	p246m14	p55o6	D13S325	y954C12	D13S327
mm12	31	NC											
mm43	50	C											
mm52	23	ND											
mm6	16	C											
mm45	50	−13											
mm50	14	NC											
mm66	14	ND											
mm5	21	ND											
mm16	30	del 13(q1q2)											
PC1	30	−13											
PC2	68	ND											
PC3	90	ND											
PC4	69	ND											
mm4	49	C											
mm17	25	NC											
mm21	63	NC											
mm24	86	del13(q14q34)											
mm26	21	−13											
mm35	25	NC											
mm36	92	NC											
mm47	14	ND											
mm51	88	−13											
mm55	72	del13(q14q34)											
mm65	94	t(12;13)(p1?1;q1?2)											
mm67	21	ND											
mm72	65	NC											
mm76	67	ND											
mm80	47	NC											
mm82	47	NC											

Legend:

monoallelic deletions

regions most likely to be deleted but not tested

no deletions

undetermined because of lack of material

significantly fewer cells deleted than with the other probes

biallelic as well as monoallelic deletions found

NC, nonclonal abnormalities.

ND, not done.

C, clonal abnormalities not involving chromosome 13.

either employed 1-3 probes for the 13q14 region or, as in one study, used noncontiguous probes spanning the whole of the 13q arm to determine the "hot spots" of deletion (Shaughnessy et al., 2000).

Previous studies have identified the 13q14 followed by the 13q21 region to be the commonest sites deleted on chromosome 13 in MM (Sawyer et al., 1995; Cigudosa et al., 1998; Shaughnessy et al., 2000). Therefore, in the present study we endeavored to delineate more precisely the CDR at 13q14–q21 by studying 82 MM cases. Consequently, a CDR of approximately 350 kb was identified, including b34f20, centromeric to *D13S319*, and p246m14, centromeric to *D13S25* (Fig. 1). The CDR identified in our series of MM cases overlaps with the CDR(s) implicated in B-CLL by several groups (Bullrich et al., 1996; Bouyge-Moreau et al., 1997; Kalachikov et al., 1997; Corcoran et al., 1998; Stilgenbauer et al., 1998). Researchers working on CLL have thoroughly analyzed this region, and have shown that a number of ESTs reside mostly within and around the 100-kb region centromeric to *D13S319* (Kalachikov et al., 1997). A CpG island was then detected at *D13S319*, and an EST (SGC32580) proximal to the telomeric border of our delineated region (Bouyge-Moreau et al., 1997). The latter EST was found to be expressed in hematologic tissue and so a possible candidate for a TSG. This was followed by the cloning of two candidate TSGs centromeric to *D13S319*, termed *DLEU1* and *DLEU2*, where deletion of the first exon of both genes was found in nearly all B-CLL patients (Liu et al., 1997). These two genes, however, were excluded as possible TSGs because neither had homology to previously published genes, both were found to have noncoding transcripts, and no mutations were detected for either gene in any B-CLL sample (Rondeau et al., 1999; Migliazza et al., 2001). A recent study reported that *DLEU1* is part of a large 560-kb gene that has multiple splice sites (*BCMS*) (Wolf et al., 2001), and suggested that *BCMS* may be a candidate TSG for B-CLL, despite all its transcripts belonging to the group of noncoding RNA. This notion was based on the alignment of *BCMS* with the different critical subregions identified in B-CLL (Wolf et al., 2001). The telomeric portion of this gene, however, extends significantly beyond the distal border of the CDR identified in our MM patients.

RFP2 (*LEU5*, *CAR*) was cloned centromeric to *DLEU1* and *DLEU2* and lies within the vicinity of our critical region. This gene is the homolog of the RET finger protein (RFP) and belongs to the coiled-coil domain, containing a subfamily of RING–zinc-finger proteins. Because of the proven link between RING-finger–containing proteins and tumorigenesis, and because it has a high degree of homology to other TSGs such as *BRCA1*, *RFP2* was suggested to be the most credible candidate within this region (Kapanadze et al., 1998, 2000; Migliazza et al., 2001).

Based on these facts, we investigated for the presence of mutations in *RFP2*. However, a thorough search revealed no mutations in any patient or cell line harboring deletions.

The question still remains as to whether *RFP2* is involved through other mechanisms, whether epigenetic such as DNA methylation or through haploinsufficiency. Migliazza and co-workers (2001) could not find diminished expression of *RFP2* in B-CLL patients through use of Northern blot analysis. In contrast, with the aid of a more sensitive quantitative real-time PCR, Mertens et al. (2002) recently demonstrated a significant downregulation of *RFP2* mRNA, independent of DNA methylation, in all B-CLL patients tested. They found that B-CLL cases with deletions at 13q14 had a 10-fold downregulation, whereas those who were disomic for this locus had a fourfold downregulation, compared with that of control B cells. These data are highly suggestive of an involvement of *RFP2* in the pathogenesis of B-CLL through haploinsufficiency, and warrant a similar investigation in MM, to elucidate whether the same applies to this malignancy.

The prevalence of 13q abnormalities in this study is similar to that reported by other investigators who used interphase FISH (Chang et al., 1999; Konigsberg et al., 2000b; Zojer et al., 2000; Fonseca et al., 2001). The only discrepancy was with the study by Shaughnessy et al. (2000), who reported a much higher incidence of 86%. However, this observation has not yet been confirmed by other researchers. Fifty-five percent of deletions in our cohort of patients were major, extending to the telomere. Although this represents a relatively high incidence, it is less than those reported in two recent studies, where 85 and 92% of 13q deletions were found to be large (Avet-Loiseau et al., 2000; Fonseca et al., 2001). The discordance between our findings and these reports may be explained by the fact that neither of these two groups tested the region between 13q14 and 13q34. Because we have shown the existence of intact chromosomal material in between these two regions in four of our patients, the use of a 13q14 probe in conjunction with a telomere 13q probe alone should not be taken as evidence of loss of the whole 13q arm, as

suggested in the previous studies. Our findings are in close agreement with those reported by Sawyer et al. (1995). By conventional cytogenetics, these authors found monosomy 13 in two thirds of cases with chromosome 13 deletions, whereas one third of them were found to have interstitial deletions, mainly involving 13q14. Shaughnessy et al. (2000) also revealed that only 40% of their cases with chromosome 13 deletions were major losses, smaller deletions being localized mainly to 13q14.

The frequent existence of large deletions in MM contrasts with studies in CLL, where almost all deletions are confined to 13q14 (Bouyge-Moreau et al., 1997; Dohner et al., 1997; Corcoran et al., 1998). This observation, along with the finding that chromosome 13 deletions do not confer a poor prognosis in CLL (Dohner et al., 1997), raises the issue of the possible involvement of an additional gene(s) distal to 13q14 in MM and possibly residing at 13q34. This gene may be responsible for the divergent clinical implications of chromosome 13 loss in the two diseases. Our finding of four cases with dual loss at 13q34 and 13q14 and an intact intervening region further substantiates this hypothesis.

We have identified a critical region of deletion at 13q14 in a cohort of MM patients and excluded the presence of mutations in the most credible TSG lying within its vicinity in a group of myeloma patients and cell lines. Further studies are necessary to address the critical issue of whether *RFP2* may be implicated in MM through haploinsufficiency, or whether another TSG within the CDR may be involved alone or in conjunction with *RFP2*.

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